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Functional Interaction of BPV-1 E2 with the Papillomavirus Genome: A Dissertation

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THE FUNCTIONAL INTERACTION OF BPV1 E2 WITH THE PAPILLOMAVIRUS GENOME

A Dissertation Presented
By
Suzanne Marie Melanson

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Virology

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Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences
Immunology and Virology Program
February 24, 2009

Dedication

I would like to dedicate this work to my family. I think I always had the ability to succeed academically, but I was easily side-tracked and suffered from a supreme lack of confidence. I watched as those around me progressed in both their personal lives and careers and always thought it beyond my reach. Then, inspired by the success of my sister, Nicole Melanson Corcoran, and great friend Michael Quintal, I finally graduated from college. I then went on to obtain a Master's degree and now, finally, my Doctorate. Without the daily support of my Mother and Father, Theresa and Robert Melanson, I would not have made it through the first year, let alone to graduation. I love you and will always cherish the memories of a cup of coffee and the morning walks with Gibson.

My sisters Michele, Renee and Nicole have been here to cheer me on, give me support, and to listen to me complain these past seven years. For that I thank them. They have been busy working, having families and raising fantastic children and all I did was get a Ph.D. It seems rather insignificant in comparison. On the other hand, it's really better than not having gotten a Ph.D. over the past seven years. Not to leave out my brother Scott, and In-laws Pat, Casey, Brandi and Ernie who were always there for me. Thanks guys. You all made it easy, well easier anyway, and a lot more fun than it would have been otherwise. Mike, Nick, Tess, Taylor, Isabel, Alex and Kian-it's never too late!!!! Go where your life takes you and you'll figure it all out along the way.

For my grandparents, I wish you could all be here to see this. Memere Melanson, I love you and wish Pepere could be here with us now. To be on your own takes strength and courage and I hope to be half as brave now as you are at 97. Memere and Pepere Scott never even saw me graduate college, but I know they've been with me every step on this long, long road. You never had more than a high school education and you were all so successful. I may never achieve that, despite all these degrees. I will always work hard and strive to be someone you can all be proud of.

Mike and Dan, thanks for all the love and support and warm desert vacations. From high school to college to grad school-I took the long road to get here. If I hadn't I might have missed you. No degree is worth that. You taught me to work hard and inspired me to be more, because you could be more. I could never ask for any greater gift.

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JM Peters for the hScc1-myc HeLa cell line.

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Abstract

The bovine papillomavirus type 1 E2 protein is a multifunctional early viral protein with roles in all phases of the cell cycle. E2 is required during G1 as a transcription factor, in S phase to initiate viral replication and during mitosis to tether the viral genome to dividing DNA. The viral genome contains 17 E2 binding sites, the majority of which are concentrated in the long control region (LCR), a regulatory region that is upstream of the viral coding sequence. The role of these binding sites has been explored *in vitro* using small plasmids and E1 and E2 proteins expressed in bacteria and insect cells. In this study we attempt to examine the placement of E2 on its binding sites during all phases of the cell cycle and in the context of a stably replicating viral system.

As part of the examination of the role of E2 during mitosis, we have also examined the role of the cohesin protein Scc1 in viral tethering. Two groups have published disparate reports identifying the cellular protein that binds to the transactivation domain of E2 to stably maintain viral genomes during cell division. Our group has published that it is the DNA helicase ChlR1 that is required for viral tethering, while it has been reported that it is the bromodomain protein Brd4 that is responsible. In this study we contribute to a report that shows that the cellular protein Scc1 binds to the viral genome through a ChlR1 independent mechanism. The cohesin protein binds to BPV-1 E2 at intermittent stages of the

cell cycle and may be a factor in viral genome tethering. This interaction may also be important for regulating viral transcription.

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Introduction

The Role of Papillomavirus in Disease

Papillomavirus is a small non-enveloped double stranded DNA virus with a circular genome of around 7900 base pairs. This tiny genome contains an average 10 open reading frames, two of which code for structural proteins, the remainder for nonstructural. Papillomaviruses are species specific and infect both birds and mammals, with over 100 types identified that infect humans alone (31). These types are classified by differences in the E6, E7 and L1 proteins with a sequence variation greater than 10% required for the classification of a new type (33). As papillomaviruses are nonenveloped they are very stable and can exist in the environment for extended periods of time, retaining 30% infectivity seven days after desiccation from cell extracts (134). Despite this stability, which lends to the possibility of infection from inanimate objects, the most common form of transmission is direct contact with an infected individual of the same species (134).

The most common result of exposure to papillomavirus is a benign cutaneous lesion, or wart, occurring at the site of infection. A wart is the result of altered growth patterns in epithelial cells. The production of viral proteins causes the epidermal layer to retain increased numbers of cells resulting in subsequent epithelial thickening (156). Most cutaneous warts develop on the hands and feet and are eventually cleared by the immune system of the infected individual.

Subsets of persons who become infected by papillomavirus are genetically pre-disposed to a condition called epidermodysplasia verruciformis (EV). The genes responsible for this susceptibility have been identified as the *EVER1* (TMC6) and *EVER2* (TMC8) genes located on chromosome 17 (133). Little is known of the function of these genes or their role in EV. Individuals with suppressed immune systems or immunological disorders may also be afflicted with EV. Persons suffering from this disease are unable to clear papillomavirus infection and will develop flat lesions on many areas of the body. These lesions will often progress to carcinoma, especially in areas of the skin exposed to sunlight, e.g., the head, neck, arms and hands. The HPV types 5 and 8 are most commonly found among patients with EV associated cancers (reviewed (122)).

In addition to epidermal infection, HPV is also capable of establishing infection in mucosal tissue. This encompasses both genital and non-genital areas. Infection by these types of HPV may result in genital warts, which again are often cleared by the host immune system. In some cases infection by these types will progress to cervical cancer. These genital-mucosal strains of HPV are often loosely classified into either the high-risk or low-risk categories. Those termed high risk are those most often associated with cancers of the cervix and include, but are not limited to, types 16, 18, 31,33, 39, 45, 52, 58. The two main types responsible for genital warts, type 6 and 11 are considered low risk and are rarely found associated with cervical carcinoma (reviewed (192)).

Those types of HPV most associated with cervical cancer, 16 and 18, are the same types most often associated with a proportion of oropharyngeal, anal,

vaginal, vulvar and penile cancers (49). Current data estimates that the numbers of cases of HPV associated non-cervical cancers in the United Sates roughly equal the number of HPV associated cervical cancers and the number of men affected by these cancers is equivalent to the number of women (49). Unlike cervical cancer, where HPV is the causative agent in nearly 100% of all cases, the correlation between HPV infection and noncervical cancer is not, with a range from 36 to 93% of these cancers being associated with HPV (49).

Worldwide, the incidence of cervical cancer is highest in those areas with inadequate health care and where the number of lifetime sexual partners is high. Developing countries account for greater than 80% of the worldwide incidence of cervical cancer (126). In 2002 the worldwide estimate for cervical cancer diagnoses was 500,000 with the number of deaths numbering 275,000 (127). Screening programs with consistent follow-up in the developed world have led to a reduction in deaths from cervical cancer. However, even in the United States there are disparities in both the incidence and mortality rates. During the five year period from 1998-2003 there were 65,074 cases of invasive cervical carcinoma with 24,707 reported deaths from cervical cancer in the United States (175). Black and Hispanic women suffered from a higher incidence of cervical cancer than did white women, with increased incidence in the southern part of the country as compared to the Northeast, Midwest and West (175). The introduction of a vaccine to combat cervical cancer may lead to reduced rates among all populations. The two approved HPV vaccines by Glaxo-Smith-Kline and Merck, have shown to have a 95% effective rate against HPV types 16 and

18 (27, 143), however the cost is roughly $360 for the three dose regimen (77). Neither vaccine has been shown to effectively lead to regression of established HPV lesions or infections (143). In developing countries and areas of the United States where many are unable to afford adequate health care the vaccine may not yet be a viable option for preventing HPV related carcinomas. The vaccine has not shown to be protective for those who have been previously exposed to the virus, nor is it effective in 100% of vaccinated individuals. There has also been shown to be little or no cross-protection achieved with the current vaccines, and as these types account for only 70% of cervical cancer cases it is important to realize there will continue to be HPV disease resulting from non-vaccine strains of the virus, necessitating continued work in the field of papillomavirus research and discovery.

The Cell Cycle and DNA Replication

The cell cycle figures prominently in the experiments undertaken in this study. The papillomavirus E2 protein is a multifunctional viral protein that has a role in all phases of the cell cycle. In G1, E2 acts as a transcriptional activator. E2 binds to viral DNA in a sequence-specific manner through the C terminal DNA binding domain of the protein (6). The N terminus serves as a transcriptional activator by binding cellular transcription factors and initiating transcription of viral proteins. E2 remains bound to the viral genome, through the DNA binding domain, during S phase and attracts the papillomavirus E1 protein to the viral origin to initiate replication of the viral genome. In preparation for mitosis E2 is bound to the viral genome, and to host factors through its activation domain, and tethers the viral genome to the host chromosomes to ensure faithful segregation of the replicated viral genomes (72, 90, 131).

Eukaryotic cells undergo alternate periods of growth, replication and division in a precisely regulated manner. The replication phase (S) is accompanied by two gap phases (G1 and G2) and a mitotic phase (M) where separation of the replicated DNA occurs (reviewed (167)). Upon differentiation or senescence the cells exit this cycle and are said be in G0. Following mitosis daughter cells are in G1. Here the cells grow, needing to double in size prior to the initiation of the DNA replication phase. Once in S phase, DNA replication initiates and will proceed only once per cell cycle. From there, the cells move

into G2, where centromere duplication occurs. DNA replication and repair must be fully complete before the cells can proceed from G2 to mitosis. Now in M phase, the newly replicated chromosomes, called sister chromatids, line up along the spindles, attached by their kinetochores, and wait for additional checkpoints to be satisfied before division is completed. Failure to line up correctly or attach to a spindle will activate another checkpoint, and mitosis will be delayed until this checkpoint is satisfied. Cells that have successfully exited mitosis find themselves in G1, ready to begin again (See figure I-1). The cell cycle is a very complex, tightly regulated system with many check points in place to keep the cell from exiting, re-replicating and dividing without all the chromosomes properly duplicated, aligned and attached. These stages of the cell cycle are irreversible and rely not on single proteins but on a complex network of transient signals that provide directionality to the cycling cell (120).

Master regulators of the cell cycle are the cyclins and cyclin dependent kinases. Through phosphorylation these proteins regulate transcription and degradation to control DNA replication, entry into and exit from mitosis. The cell cycle begins with activation of cyclins in cells in G1 (Figure I-1). Cells are normally induced to begin replication through activation by mitogenic signaling through the Ras complex (69). This signaling activates cyclin D and cyclin E which upregulate Cdk4/6 and Cdk2 respectively (37, 79, 121). When cells are quiescent, these kinases are inhibited by members of the cyclin kinase inhibitors (CKI) family, which includes the proteins $p27^{KIP1}$ and $p21^{CIP1/WAF1}$ (39, 56, 62, 182). Upregulation of these kinases leads to hyper-phosphorylation of the

retinoblastoma protein (pRb), early in G1 by cyclin D/Cdk4, and late in G1 by cyclin E/Cdk2 (32, 101, 108, 110) (Figure I.1). This results in release of repression by the transcription factor E2F, and an increase in transcription of factors required for S phase. These factors include elements directly required for replication such as Orc1, Cdc6, and DNA polymerase alpha (128, Ohtani, 1996 #186, 184), as well as for maintaining levels of factors such as cyclin D and E to ensure progress through S phase. Cyclin E is upregulated by pRb, as is cyclin A, both of which are required for transition through S phase (38). Cyclin A/Cdk2 maintains phosphorylation of pRb throughout S (38).

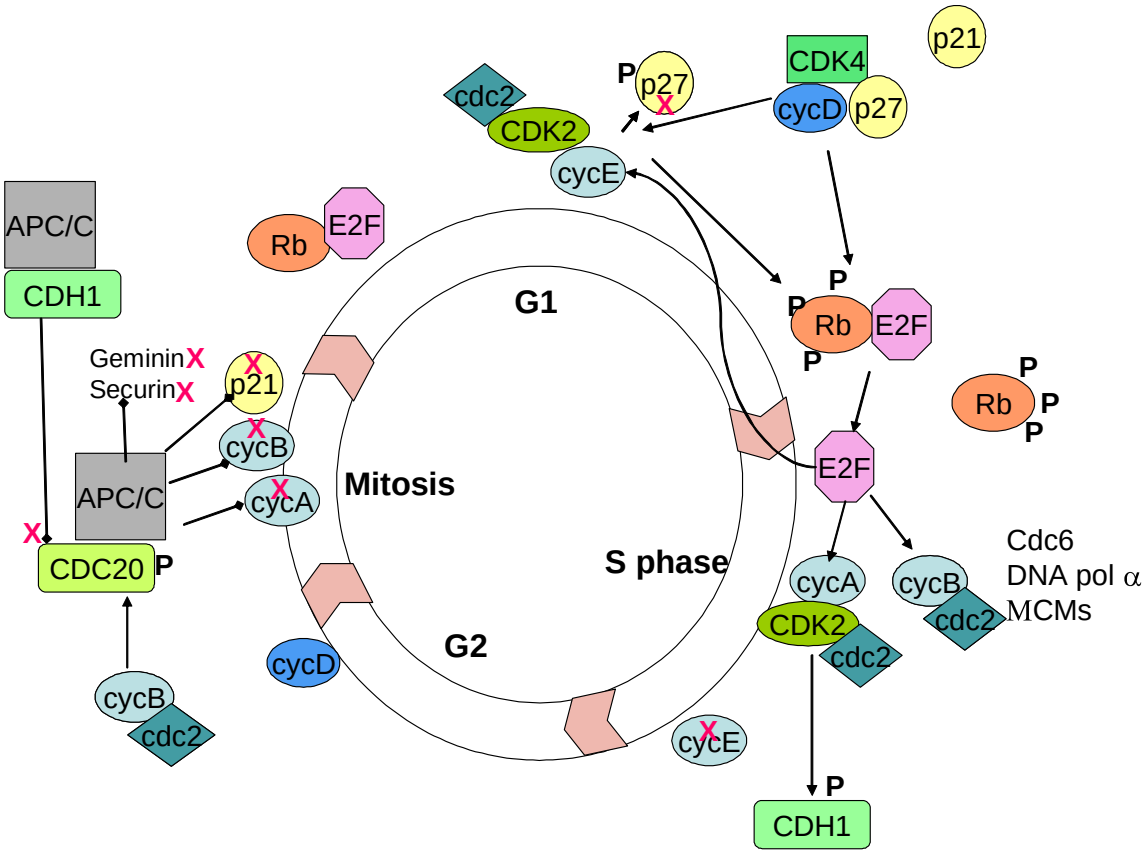
In addition to blocking the progression of S phase by inhibiting transcription of essential factors, pRb also blocks replication by binding to the pre-replication complex (pre-RC) (51). Mammalian DNA replication requires the formation of the pre-RC factors on the origin to enable the initiation of replication. The origin recognition complex (ORC) is a 6 member structure that is a component of the pre-RC. The protein CDT1 then binds to ORC and recruits the six member DNA helicase component of the replication complex, the minichromosome maintenance proteins (MCM). MCM proteins 2-7 are loaded at the origin by the factor CDC-6 (76).

An additional member of the MCM family is required for replication. MCM10 is loaded onto DNA at the origin and CDC45 is then also added to the complex. This initiates unwinding of the DNA and allows replication protein A (RPA), a single stranded (SS) DNA binding protein, access, to stabilize the SS DNA (76). CDC45 is required for elongation of DNA synthesis and its

association is followed by the addition of PCNA, which is loaded with the aid of replication factor C, and DNA polymerases δ and ε. Cyclin A/Cdk2 remains associated with the replication fork, is required for elongation, and is known to phosphorylate PCNA, pol α and pol δ (183).

Once S phase has begun and the origins of replication have fired, the cyclin-CDK complexes function to inactivate the proteins required for initiation in order to prevent re-replication. Cdc6 is phosporylated by cyclin A/Cdk2 during S phase resulting in transport of the initiation factor into the cytoplasm, in order to prevent re-replication (28). Phosphorylation and activation of MCM is also controlled through cyclinA/Cdk2 (74). Following initiation of replication MCM is removed from chromatin by the movement of the replication fork and is not able to bind again without the licensing factor Cdc6. CDT1 is expressed only in G1 and S, and its activity is inhibited by the protein geminin, which is expressed only in S and G2 and is degraded late in mitosis (93, 104). Cyclin E is also degraded during S to prevent another round of replication (112).

Figure I.1. **The Cell Cycle.** A representation of the mitotic cyclins and their roles in the cell cycle. The phases of the cell cycle are shown in the center. See text for additional information.


p21
CDK4
P
p27
cdc2
cycD
p27
X
CDK2
cycE
APC/C
Rb
E2F
CDH1
G1
P
P
Rb
E2F
P
Geminin
X
Securin
X
X
p21
P
Rb
P
P
X
cycB
E2F
APC/C
Mitosis
X
cycA
X
S phase
Cdc6
DNA pol α
MCMs
CDC20
P
cycA
cycB
CDK2
cdc2
cdc2
cycB
cdc2
G2
cycD
X
cycE
P
CDH1

Cells progressing through S phase are unable to re-initiate replication and enter G2. As they do, cyclin E is degraded, downregulating E2F and transcription of factors required for S phase. During late S phase cells begin to accumulate Cdc20. At the onset of mitosis, phosphorylation of the anaphase promoting complex/cyclosome (APC/C) by cyclin B/cdc2 and polo-like kinase (PLK) activates the ubiquitination function of the APC/C^{CDC20}, resulting in the degradation of both cyclin A and cyclin B in pro-metaphase/metaphase (52). The cellular protein securin is also degraded by the APC/C^{CDC20} allowing for the action of the protein separase, which cleaves the Scc1 protein allowing for mitotic segregation of sister chromatids (59, 165). The geminin protein is degraded here as well, releasing the Cdt1 protein and allowing for another round of replication licensing, as does the degradation of the PKI p21 (4, 104). Upon degradation of cyclin A/Cdk2 the APC/C^{CDC20} complex is replaced by the APC/C^{CDH1} following mitosis. This complex completes the degradation of cyclin B and promotes the repression of S phase by degrading factors that ubiquitinate $p27^{kip1}$ and $p21^{cip1/waf1}$ allowing them to delay progression into S phase until G1 is complete (Figure I.1) (80).

In order to examine the assembly of factors at the viral origin, cell cycle check points can be chemically induced by the addition of excess thymidine. Thymidine is phosphorylated to dTTP, which blocks the reduction of CDP by ribonucleotide reductase leading to a decrease in dCTP (13, 41). This decrease results in replication forks stalled at the initiation of replication and activates both

the ATM and ATR pathways of DNA repair response blocking the cells in the very early stages of S phase (14). This checkpoint activation provides a useful tool for examining viral proteins locked at the origin of replication, and is used extensively in this thesis.

Aneuploidy, the incorrect number of chromosomes in a cell, is commonly found in tumor cells and tumor cell lines. Cells have a method to combat this, which is the mitotic checkpoint, or spindle assembly checkpoint (reviewed in (161)). As cells approach prometaphase (shown above) mitotic spindles form and the paired sister chromatids align and attach to the mitotic spindles by their kinetochores. If all the chromosomes fail to properly align or attach to a spindle, the cells fail to progress to anaphase. If all is well and the checkpoint is not activated, the anaphase promoting complex/cyclosome (APC/C) will bind Cdc20, target Securin and Cyclin B for destruction and release separase to cleave Scc1, the member of the cohesin complex that binds the sister chromatids together (17, 26, 29). The cells will then proceed through anaphase to exit mitosis through telophase and return to G1. In this study we use the chemical nocodazole to block formation of microtubules in prometaphase, thus inducing the mitotic checkpoint and allowing examination of viral protein interactions at the onset of mitosis. In the final chapter we will also introduce interactions between the papillomavirus E2 protein and members of the cohesin family.

Papillomavirus Life Cycle

The infectious papillomavirus particle is a non-enveloped icosahedral capsid 60 nm in diameter. Papillomavirus genetic information is encoded on an 8 kb circular genome that can be divided into three regions. The first is the non-coding long control region (LCR) or upstream regulatory region (URR); the early region encodes genes E1, E2, E4, E5, E6, and E7; and the third region encodes the late major and minor capsid proteins, L1 and L2.

The initial papillomavirus infection is believed to occur in the epithelium through micro-abrasions, allowing for the establishment of the infection in the dividing layer of basal cells. These cells initiate as stem cells, become transit amplifying cells which divide several more times, eventually withdrawing from the cell cycle in the basal layer and entering into a differentiation pattern (176). Infection of this cell type leads to establishment of the viral genome as a stably replicating extra-chromosomal element, or episome (88), believed to occur through a high output viral genome replication event. Episomes then replicate at a low copy number along with the cellular genome during S phase of the cell cycle, and faithfully partition to daughter cells upon division. The expression of viral proteins E1 and E2 is required for the initiation of viral replication and establishment and maintenance of infection in dividing basal cells (53). As these cells differentiate the E7 protein maintains them in a replication competent state (20). The virus then begins a program of vegetative genome production, coupled with expression of late viral proteins, and assembly of virions, and is shed as the differentiated cells move to the surface. The mechanism for this switch in viral

replication from initiation of infection to maintenance of a stable copy number, then to high-output genome reproduction, is not yet fully understood.

The role of the viral proteins E1 and E2 in the viral life cycle will be covered in depth in later chapters. The role of E6 and E7 will be discussed briefly here. Papillomavirus encodes only 2 proteins known to be required for replication, those are the E1 and E2. The majority of the factors required are cellular proteins. In order for the virus to continue to replicate in differentiating cells it is important that these cells continue to express components of the DNA replication machinery. Papillomavirus E6 and E7 proteins maintain the differentiating cells in cycle so that vegetative replication and virion production can occur (45). The retinoblastoma protein (pRb) binds to a member of the E2F transcription family during G1 effectively blocking induction of proteins required for genomic replication and inhibiting entry into S phase. Phosphorylation of Rb by cyclin dependent kinases releases E2F, allowing for cell cycle progression (reviewed (166)). E7 binds hypophosphorylated pRb, interrupting this cycle of regulation and allowing for transcription of genes required for replication (116). BPV-1 E7 has been shown to bind pRb only weakly (116), however it does activate c-Myc, leading to activation of cyclin-A and cyclin-E related kinases as an alternate method to maintain cells in a replication competent state (42).

Human papillomavirus E7 protein has primarily been shown to be a viral oncogene required for immortalization and transformation by high risk papillomaviruses (130, 173). This is primarily due to its interactions with pRb family members. The high risk papillomavirus E6 protein is also required for

transformation of host cells. E7 alone can immortalize human keratinocytes, but the presence of E6 increases the frequency of these events (9, 60). Papillomavirus E6 plays a major role in the extension of the replication window for the virus. E6 binds the proteins E6AP, an E2 ubiquitin ligase, and p53 and through the proteasome will degrade the p53 protein (139, 140). The presence of p53 blocks progression to S phase and its degradation abrogates the G1/S checkpoint allowing the cell cycle to progress. P53, however, functions to cause growth arrest or apoptosis in cells in response to stress, DNA damage or un-programmed entry into the cell cycle (138, 180) and as such is considered to be a tumor suppressor. Loss of this tumor suppressor function leads to the possibility of continued replication of mutated genomes, furthering the transformation process. While expression of E6 and E7 alone can immortalize human keratinocytes, transformation requires significant passaging, during which time an additional transforming event is believed to occur (reviewed (43, 65)). Regulation of transcription of E6 and E7 requires the papillomavirus E2 protein. Disruption of this protein, through mutation or integration of the viral genome into host DNA, is often found in HPV transformed cells (75) and may help initiate the immortalized phenotype through over-expression of E6/E7, leading eventually to HPV related cancers.

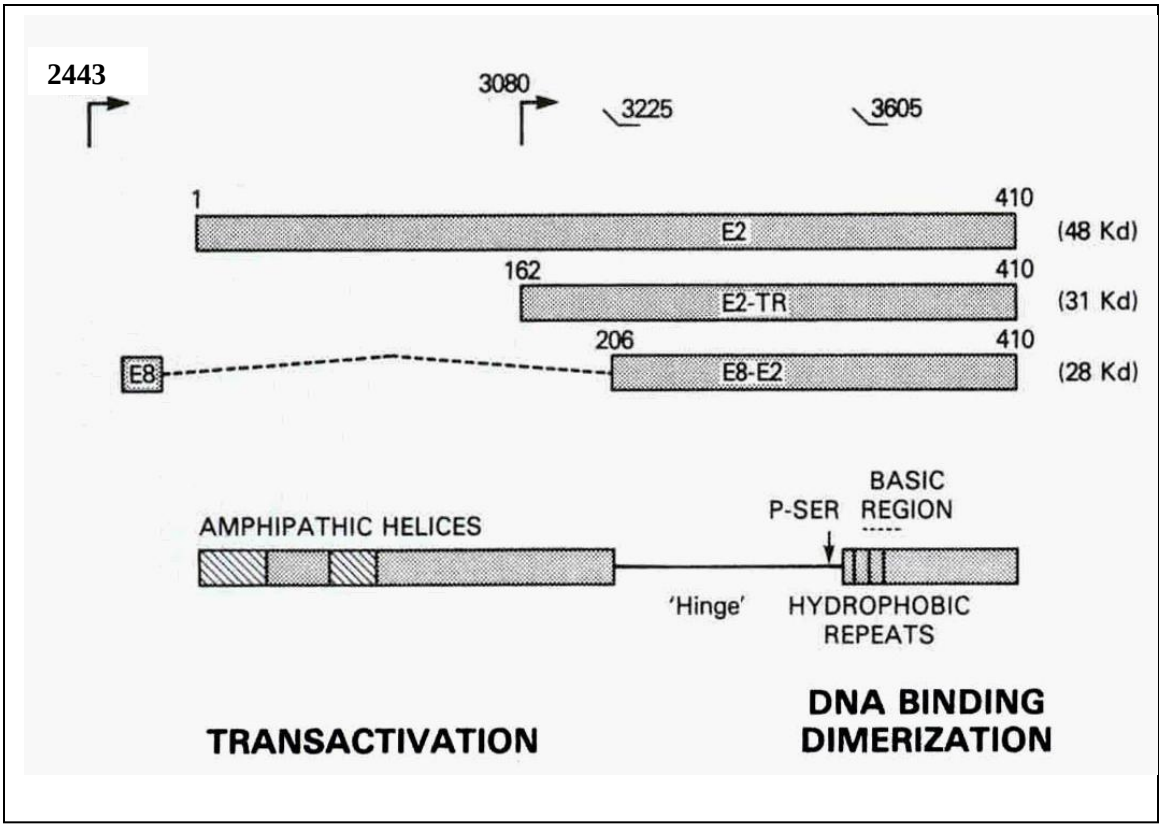
The E2 Protein

The papillomavirus E2 protein is a product of viral early gene expression. The BPV-1 E2 open reading frame (ORF) codes for a transactivation competent form of E2 (E2 or E2TA) which has a molecular mass of about 48 kDa, and two repressor forms of E2; E2R, or E2TR and E8/E2, with molecular masses of 31 and 28 kDa respectively (70,(87). Papillomavirus E2 proteins are comprised of 3 distinct functional domains (see Figure I.2). The N terminal region is necessary for interacting with proteins required for transcriptional activation, viral genome segregation, and DNA replication. The C terminal is required for sequence specific DNA binding (6), and there is an intermediate region, referred to as the hinge region, which allows for flexible binding to both the DNA and N terminal targets (50). Full length E2 (amino acids 1-410) encodes for all 3 of these regions. The E2R protein (also known as E2-TR) contains only the DNA binding domain and includes amino acids 162-410 initiated from an internal methionine (85). The E8/E2 protein is composed of amino acids 206-410 preceded by 11 amino acids from the E8 ORF (87). The N and C terminal domains are well conserved among papillomaviruses and their crystal structures have been determined (63, 66). The hinge region is not well conserved across papillomavirus species and to date no structural data is available.

The regulation of the E2 repressor proteins is controlled from distinct promoters located at nucleotides 3080 (E2R) (85) and 890 (E8/E2) (22). Initially transcription of full length E2 was shown to be directed from a promoter located in the region of nucleotide 2443 (68, 187). However, it has since been shown

that promoters at 890 and 7940 also regulate expression of BPV E2TA (172). E2 regulates the production of its own transcripts through both activation and repression of its promoters.

Figure I.2 . **Cartoon structures of the BPV-1 E2 protein**. To the right are listed the molecular weights of the proteins. The bottom panel is a schematic of the 3 regions of E2, the N terminal transactivation domain, the C-terminal DNA binding domain and the non-conserved flexible hinge region joining the two. Bent arrows are the promoters which regulate transcription of these gene products. Transcription of E2 also initiates at P890 and P7940 (Not shown in figure) (103).


2443
3080
3225
3605
1
410
E2
(48 Kd)
162
410
E2-TR
(31 Kd)
206
410
E8
E8-E2
(28 Kd)
BASIC
P-SER
REGION
AMPHIPATHIC HELICES
'Hinge'
HYDROPHOBIC
REPEATS
TRANSACTIVATION
DNA BINDING
DIMERIZATION

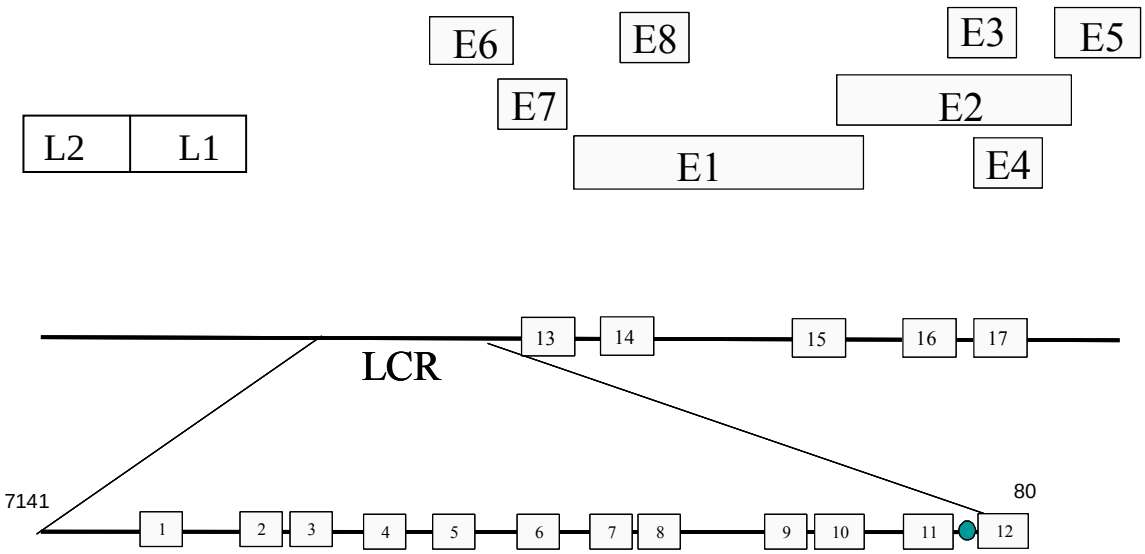
E2 activates transcription by binding viral DNA with its C-terminal DNA binding domain and transcription factors with the N-terminal transactivation domain (102). Only binding of transactivation competent E2 homodimers will result in activation of transcription. Interaction of E2 with factors such as p300, GPS2/AMF-1 and Brd4 have all been shown to contribute to E2 mediated transcription of viral proteins (16, 105, 115). Transcriptional activation occurs through binding of E2 sites adjacent to the promoter (153) as well as through binding to distal sites in the genome (163). Activation through binding distal to the E2 promoter can be weak, but is strengthened through addition of binding sites or addition of a single binding site adjacent to the promoter. Bending of bound DNA is believed to be required to bring the proximal and distal sites together and initiate activation (163).

Repression of transcription by full length E2 also occurs. When expressed at high levels, E2 will bind to lower affinity sites denying access to cellular factors, such as Sp1 and TFIID, required for activation (159). Preferential binding to higher affinity sites will lead to transactivation in the presence of low levels of E2. E2-E2R heterodimerization also results in a repressor function for E2 (8).

E2 recognizes a specific DNA sequence, $ACCN_6GGT$ (6) which is present 12 times in the bovine papillomavirus genome, with 5 sites that display similar consensus and also bind E2 (Figure I.3)(94). Of these 17 sites the majority are located in the BPV-1 LCR and their affinities for the E2 consensus sequence

varies 300 fold. The number of E2 binding sites also varies among papillomaviruses, with HPV 16 and 18 genomes only containing 4 binding sites, all located in the viral LCR. Both full length E2 and the E2R protein bind a single recognition sequence as homodimers through the C-terminal DNA binding domain which is also the region required for dimerization (36, 114).

Figure I.3. **E2 Binding Sites in the BPV1 Genome.** The E2 binding sites in the BPV1 genome are labeled 1-17. The origin of replication and E1 binding site is marked by a filled circle. The open reading frames in BPV-1 are shown above.


E6
E8
E3
E5
E7
E2
L2
L1
E1
E4
13
14
15
16
17
LCR
7141
80
1
2
3
4
5
6
7
8
9
10
11
12

In addition to its role in transcription, E2 also has roles in initiation of viral replication and in localizing the viral genome to genomic DNA during cell division. Activation and replication are separate and distinct functions of E2, and although increased transcription of viral proteins also leads to increased replication in transient replication assays, the transactivation function of E2 is not required for replication (178). Additional roles of E2 will be discussed further in later chapters.

Preface

A portion of this work will be submitted for publication.

Topography of Bovine Papillomavirus E2 Protein on the Viral Genome During the Cell Cycle.
Suzanne M. Melanson and Elliot J. Androphy

Chapter I:

Topographic Localization of Bovine Papillomavirus E2 Protein During the Cell Cycle

The multi-functional papillomavirus E2 protein plays important roles in viral replication, transcriptional activation and genome maintenance while the viral E1 protein, a replicative helicase, functions solely in the initiation of viral replication. The bovine papillomavirus genome contains a single E1 binding site at the origin of replication, in addition to seventeen sequence specific E2 binding sites largely concentrated within the long control region. In this study we have examined the association of both E2 and E1 with their specific binding sites throughout the cell cycle utilizing a murine cell line that stably expresses episomal bovine papillomavirus genomes. Using chromatin immunoprecipitation (ChIP) followed by restriction enzyme digestion and PCR, we were able to determine the E1 and E2 binding site occupation during different phases of the cell cycle. BPV E1 was found located only at the origin and on replicating DNA, while BPV E2 remains associated with specific sites on the genome throughout the cell cycle.

The Papillomavirus E1 Protein

The papillomavirus early proteins E1 and E2 have been shown to be the only viral proteins required for transient replication of the genome (111, 146, 171). The E1 ORF is the largest in the 8 kb papillomavirus genome and is also the most highly conserved, producing a protein that averages about 650 amino acids with molecular weight in the range of 68-76 kDa. E1 functions as an ATP dependent replicative helicase that binds, unwinds, and replicates the viral DNA (reviewed (148, 157, 164, 185). E1 has been found to have three distinct domains: the N terminal, whose function has not been clearly defined in HPV, a central linker region, and a highly conserved C-terminal region. All of the functions required for DNA replication for human papillomaviruses have been mapped to the C terminal domain (44, 162). In BPV1 these functions appear to spread across the 3 domains of E1. The crystal structure of the DNA binding domain of BPV1 and HPV18 E1 have been determined (7, 40), and for HPV18 the structure of E1 in complex with E2 has also been resolved (2). Additional structures for BPV1 E1 have yet to be determined. As there seems to be a reasonable difference between BPV1 E1 and HPV E1, for simplicity, and since the experiments in this thesis use a BPV1 model, the structural data referenced will be for BPV1 E1, unless otherwise stated.

The location of the DNA binding domain of BPV1 E1 has been published by several groups (19, 92, 164). Although there has been conflicting data published, it is most likely that the DNA binding region lies between amino acids 121 and 311. In addition to its DNA binding, E1 also specifically binds BPV1 E2.

This binding requires three distinct regions of E1, two that bind the E2 transactivation domain (TAD) and one that binds the E2 DNA binding domain (DBD) (10, 11, 19, 113). The purpose behind the multiple E1-E2 interactions appears to be the generation of specificity of E1 binding. The E2 DBD is sequence specific and it binds to its site at the origin of replication, bringing the low affinity DBD of E1 to the DNA. The TAD of E2 is thought to bind the helicase domain of E1, preventing that region from binding the DNA non-specifically, but with higher affinity, until it's required (157, 158).

In a cell free system E1 can initiate DNA replication in the absence of the viral E2 protein (99) and while E1 is capable of binding DNA with little or no sequence specificity, DNA binding to the BPV1 origin is stimulated by the interaction with E2, and required for DNA binding *in vivo* (99, 100, 146, 147, 170, 186). The current model suggests that E1 and E2 bind cooperatively to the viral origin of replication, with E1 binding as a head-to-head monomer, then as a double trimer. E1 hydrolyzes ATP and displaces E2, then begins to melt and unwind the DNA while additional E1 molecules are attracted to form the double hexameric complex at the origin (135, 144).

E1 has been shown to interact with several cellular replication factors. Topoisomerase 1, a factor required for relieving torsional stress incurred by the unwinding of DNA, has been shown to interact with both E1 and E2 and to enhance binding of E1 to the viral origin, although the exact mechanism of action is not yet clear (24, 25, 70). E1 has also been shown to interact with replication protein A (RPA) a single-strand DNA binding protein required for replication on

the lagging strand of DNA (61, 107). DNA polymerase α primase, required for primer synthesis, has been shown to interact with E1 (15, 125, 154). DNA polymerase δ, theorized now to be required mainly for lagging strand replication (reviewed (84)), but known to share the majority of replication responsibilities with DNA polymerase ε, has also been shown to be required for BPV DNA replication, as have replication factor-C (RF-C), the clamp loader, and PCNA the sliding clamp (107).

While the minichromosome maintenance protein complex (Mcm2-7) and origin recognition complex (Orc) have been shown to be required for cellular DNA replication, neither protein has yet to be shown to be required for papillomavirus genome replication. It has been reported that the HPV E6 protein from multiple types binds to the Mcm7 protein subunit and can induce its expression, as well as degrade it through the E6AP ubiquitin ligase (81, 83, 149). It is not known if cellular licensing factors, such as Cdt1, play a role in papillomavirus DNA replication. However, bovine papillomavirus replicates in a random choice method where some genomes are duplicated multiple times per S phase and others not at all (48, 118). This would seem to indicate that the virus has found a method to undermine the licensing process.

BPV1 E2 and Viral Replication

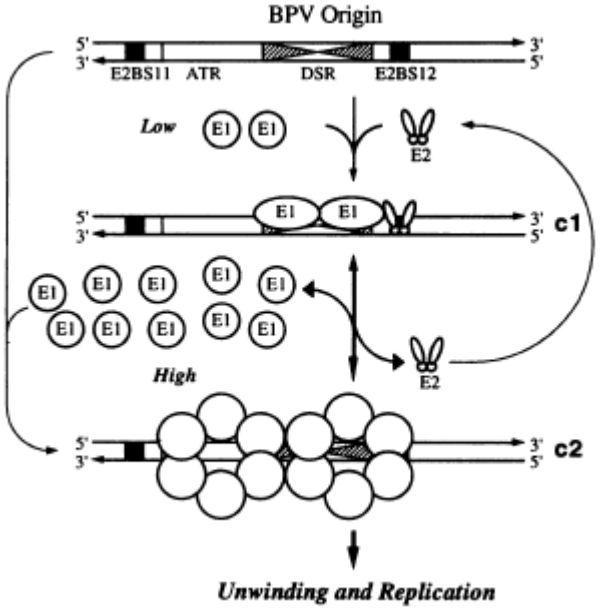
The role of E2 in transcription and its interactions with E1 have been previously discussed. However there are some additional points which will now be addressed regarding BPV1 E2 and viral replication.

The bovine papillomavirus type 1 (BPV1) has been widely used as a model system for the study of replication and maintenance in PV. When expressed in murine cell lines grown in monolayer, the BPV1 genome is able to persist episomally, displaying a stable copy number and expressing the essential early viral proteins. Comparatively, the study of episomal human papillomaviruses (HPV) can be more problematic, with the majority of commonly used cell lines containing integrated HPV sequences. The murine C127 cell line can be transformed by the BPV1 genome, leading to the expression of only extrachromosomal viral DNA, allowing for examination of replication without contamination from integrated genomes (88). The mechanism of this rodent cell transformation by BPV1 is still not fully understood, however the BPV1 E5 and E6 proteins can transform these cell lines (12, 142).

Studies of the BPV1 genome identified 17 sequence specific E2 binding sites (94), the majority of which are clustered in the long control region (LCR), also called the upstream regulatory region (URR) of the viral genome. Several binding sites are also found outside the LCR within the coding sequences of both the E1 and E2 genes. Binding sites 11 and 12 (BS11 and BS12) flank the viral origin of replication, and as such they have been extensively examined. In 1993 Lusky, *et al* used cloned fragments of LCR in EMSA to determine that either

BS11 or BS12 were required for cooperative binding of BPV E1 and E2 to the origin of replication (99). They also showed that increasing the distance between BS12 and the origin, from the wild type 9 nucleotides to 12, decreased association of E1 with the origin, while reducing from 9 to 6 nucleotides had no effect on E1/E2 cooperativity. Using baculovirus produced E1 they went on to show that in the presence of the intact BS12 E2 enhanced binding of the E1 protein 40 fold and induced unwinding of the DNA (148). In 1994 this same group demonstrated that 2 separate complexes could be isolated on the origin *in vitro* in the presence of plasmid DNA and baculovirus protein. The two complexes that were discovered were E1 and E2 dimers together, or E1 multimers. This gave rise to a model similar to the current model for origin assembly where E2 is not maintained at BS12 following initiation of viral replication (see figure 1.1) (100).

Figure 1.1 Origin Binding of BPV1 Replication Proteins An early model proposed by Lusky, *et al* 1994 (100), detailing the positioning of E1 and E2 on the viral origin with removal of E2 required for replication.


BPV Origin
5'
3'
3'
5'
E2BS11
ATR
DSR
E2BS12
Low
E1
E1
E2
E1
E1
c1
High
E2
c2
Unwinding and Replication

While the above work was being accomplished *in vitro,* Ustav, *et al* was asking the same questions using an *in vivo* system. Using transient transfection of plasmid DNA in CHO cell lines expressing E1 and E2, they were able to show that high affinity E2 binding sites at a distance from the origin of replication were able to initiate transient replication in addition to the low affinity BS12 located adjacent to the Ori (170).

In addition to its roles in replication and transcriptional regulation, E2 is required for securing the viral genome to mitotic cellular DNA (72, 90). This process ensures perpetuation of papillomavirus infection in dividing cells and cell culture systems by preventing loss of episomes through dissociation during breakdown of the nuclear envelope in mitosis (90). Proper segregation of the viral genome during mitosis requires both *cis* and *trans* factors. The *cis* requirement for proper distribution of the episome consists of a region of the viral DNA containing a cluster of E2 binding sites termed the minichromosome maintenance element (MME) (3, 90, 131). The MME is located in the region of viral nucleotide 7600. The E2 protein serves as the *trans* element and binds the viral genome through its C-terminal DNA binding domain, while additional cellular factors that facilitate genomic distribution bind to the N-terminal transactivation domain (3). The nature of these additional factors remains unresolved; our lab has reported that ChlR1, a DNA helicase, is required for the stable maintenance of the papillomavirus genome (123). Other labs have reported that introduction of a dominant negative mutant of the bromodomain protein Brd4, a protein

reported to play a major role in regulation of E2 mediated PV transcription, (105, 145, 179), results in disassociation of the viral genome from chromatin (190),

In this study we used a modified ChIP assay to elucidate occupancy of E2 consensus sequences on the BPV1 episome in a cell line that stably expresses the viral genome. Published data suggests that E2 is released from the genome following replication; however the question would then be what happens to E2 between G1/S and mitosis? We asked whether E2 association with its binding sites varies during the cell cycle.

We analyzed binding of E2 to its genomic sequences using chromatin immunoprecipitation (ChIP) of cross-linked protein/DNA complexes in synchronized cells. In addition, the association of Brd4 with the viral genome through the course of the cell cycle was also examined.

Using the replication factors known to be required transiently by BPV *in vitro* and those known to be required for Epstein Barr virus replication, we attempted to use a standard ChIP assay to compare the proteins isolated at the viral origins at the onset of S phase. Since EBV replication is restricted to once per cell cycle, it would interesting to see if licensing factors known to restrict cellular DNA replication were responsible for limiting EBV genome replication and not papillomavirus. The pre-replication complex (pre-RC), the origin recognition complex (ORC), and the mini-chromosome maintenance complex (Mcm) have all been shown to be factors required for EBV replication (18, 34, 141).

In some of these experiments our ChIP protocol was modified to include restriction enzyme digestion following the immunoprecipitation. Digestion was undertaken with endonucleases targeting specific sites between E2 binding regions in the LCR sites and throughout the remainder of the viral genome. Then, specific polymerase chain reaction (PCR) primers were used to differentiate the bound regions of the DNA. The original protocol utilizes only sonication to stochastically disrupt genomic DNA. Our modification provides specific discriminate DNA sectioning and allows for finer resolution of binding site usage compared to sonication alone. With this assay, we were able to show binding of E1 and E2 to specific regions of the episome in different phases of the cell cycle and to locate E1 in the region of the origin at initiation of viral replication.

Materials and Methods

Cell culture. The C127 A3 cells were a generous gift from M. Botchan. ID13, A3 and C127 cells were all cultured at 37°C in Dulbecco's Modified Eagle Medium (Gibco) with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml) in 5% CO_2. 1.4 X 10^6 A3 cells were plated in a 10 cm dish and 24 hours later were synchronized using 5 mM thymidine (Sigma) for 12 to 16 hours (G1/S) or 100 ng/ml nocodazole for 12 hours (M).

Chromatin immunoprecipitation (ChIP). ChIP was performed using a protocol modified from Upstate Cell signaling. A3 cells were synchronized overnight and cross-linked by the addition of formaldehyde (Sigma) (final concentration of 1%). Cells were incubated for 10 minutes at 37ºC, and washed with cold phosphate buffered saline (PBS) and scraped into a 1.5 ml collection tube. Nocodazole synchronized cells were collected using mitotic shake-off washed with cold PBS and centrifuged at 1000 g. Cells were resuspended in 250 μl lysis buffer (1% sodium dodecyl sulfate (SDS), 10mM EDTA and 50 mM Tris-HCl, pH 8.1) and incubated on ice for 10 minutes. Lysed cells were sonicated for 50 seconds in 10-second pulses at 30% output (Fisher Scientific dismembrator), and then centrifuged at 14000 g for 10 minutes at 4°C. The supernatant was removed and diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167mM NaCl, PMSF, Roche complete protease inhibitor). The ChIP lysate was pre-cleared by the addition of 30 μl protein A agarose bead slurry (Upstate Cell Signaling) and incubation at 4°C,

with agitation, for a minimum of 1 hour. After centrifuging, the supernatant was removed and added to 50 μl fresh beads containing 5 μl II-1 rabbit anti-E2 antibody, 10 μl rabbit pre-immune serum, 3 μl anti-EE ascites, 20 μl monoclonal B202 supernatant, or 10 μl anti E1 rabbit Ab. Following overnight incubation at 4°C beads were pelleted at 4000 rpm and washed with cold low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris HCl, pH 8.1, 150mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris HCl, pH 8.1, 500mM NaCl), LiCl wash buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris HCl, pH 8.1), and twice with cold TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Complexes were then released from the beads using two 15 minute incubations with 250 μl elution buffer (1% SDS, 0.1M $NaHCO_3$). The eluates were combined and reverse crosslinked by incubation with NaCl (final concentration of 0.2M) at 65°C for 4 hours. Samples then were digested with 20 μg proteinase K (Invitrogen) at 45°C for 1 hour with the addition of EDTA to 0.01M and Tris HCl, pH 6.5, to 0.04M. DNA was recovered using phenol/chloroform/isoamyl alcohol extraction and overnight isopropanol/ethanol precipitation at -20°C. DNA was re-suspended in water and analyzed by PCR.

For restriction enzyme digestion coupled ChIP, samples were cross-linked with 0.33% formaldehyde and ChIP was performed as described above. Following immunoprecipitation and washing, samples were resuspended in 100 μl of the appropriate restriction enzyme digest buffer, BSA and either 3 μl of *BanII* (New

England Biolabs), 1 μl high concentrate *BglI* (Promega), 1μl of both *BglI* and *PflM1* (New England Biolabs), or no enzyme. Restriction digests were then incubated at 37° overnight. Following digestion, samples were washed twice with TE buffer and the sample recovery continued as described above.

Re-ChIP. Sequential Re-ChIP. ChIP with restriction digest was initiated as previously described and continued through washing. Wash buffer was removed and 60 μl 1% SDS $NaHCO_3$ elution buffer added. Buffer and beads were removed to a fresh PCR tube and eluted in a PCR machine at 68º C for 10 minutes. The machine was paused every three minutes for gentle agitation of the samples. ChIPs were centrifuged at 3000g for 5 minutes, then the supernatant was removed and 950 μl of ChIP binding buffer added (recipe as described above, but without SDS) along with 30 μl Sepharose beads. Samples were pre-cleared for 1 hr at 4ºC with rotation. Agarose beads were precipitated at 1000g and supernatants transferred to new 1.5 ml tubes. While pre-clearing, the secondary Ab was bound to beads at 4º C using binding buffer without SDS and the appropriate concentration of secondary or control antibody. The supernatant was removed from the pre-bound Ab/bead mix and the pre-cleared ChIP added. Tubes were incubated ON at 4º C with rotation. ChIPs were washed 1X each with low salt wash buffer, high salt wash buffer, LiCl buffer and 2X with TE buffer. The standard protocols for elution, reverse cross-linking and DNA purification were followed following the washes.

Subtractive Re-ChIP. ChIP was initiated as described for ChIP with digestion. Following ON digestion supernatant was removed and beads discarded. Supernatant was pre-cleared with beads for 2 hrs at 4º C. The agarose beads were pelleted and the supernatant removed to a new tube. Fresh agarose beads along with the appropriate antibody were added and the samples were incubated 4º C ON with rotation. ChIPs were then washed, eluted, reverse-crosslined and the DNA was purified as previously described.

Antibody crosslinking. Protein A Sepharose beads (Upstate) were washed two times with PBS and 5 µl antibody per 1 ml bead slurry were added and mixed with rocking for one hour at room temperature. The beads were then washed two times with 10 volumes borate buffer (0.2M pH 9) and were resuspended in 10 volumes borate buffer with the addition of powdered dimethyl pimelimidate dihydrochloride (DMP) to 2 mM (52 mg/10 ml). Antibodies were incubated another 30 minutes at room temperature with rocking. The reaction was stopped by the addition of 0.2 M ethanolamine, pH 8. The beads were pelleted to remove the stop solution, then washed again with 0.2 M ethanolamine. Washing was followed by incubation for two hours at RT, with rocking, in ethanolamine. Crosslinked beads were then washed twice with PBS and stored at 4 ºC.

PCR Primers for Topographic mapping experiments.

Primer	Sequence		Extension Temp for PCR
Origin Primer Set A	Forward Reverse	tcaaaatgcagcattatattttaagct tggaacaccttgccaaagtcttc	52 ºC
Upstream E2 Binding Region Primer Set B	Forward Reverse	acacccggtacacatcctgt ctgtctgtagtttaaggcg	50 ºC
BPV LCR Primer Set C	Forward Reverse	aaagtttccattgcgtctgg gctttttctagttagctggctatttt	54 ºC
BS 16 and 17	Forward Reverse	ggtggtagaggtggagtttgatg agtagtagagcccagttccgtcag	54 ºC
L1	Forward Reverse	atctccctccaacccctgtaag gcctgtttgtttcctgtcatctg	50 ºC

The PCR conditions were primer dependent. The required number of cycles often varied between antibodies. Primer Set C most commonly averaged 25 cycles, with E1 requiing 2-5 more cycles to visualize product, and nocodazole

blocked cells also requiring additional PCR cycles. The negative control samples were always run with the highest number of cycles used for any condition.

FACS. A3 cells were synchronized as described and then fixed for flow cytometry. Briefly, thymidine blocked cells were washed with PBS and trypsinized to collect. Trypsinized cells were washed once in DMEM with FBS, twice in PBS, and centrifuged at 1000g. The cell pellet was re-suspended in 200μl ice cold PBS and gently agitated while 800 μl ice cold 100% ethanol was added drop-wise and left at 4°C overnight to fix. Following fixation cells were centrifuged at 1500 rpm for 5 minutes and fixation buffer was removed. Samples were resuspended in 1ml of propidium iodide (PI) staining solution (glucose 1g/L, RNAse A 100U/ml, propidium iodide 0.05 mg/ml in PBS sample buffer (PBS, glucose 1g/L)). Prior to FACS analysis (Becton Dickinson) samples were incubated in the dark at room temperature for 30 minutes and filtered through gauze. Data was analyzed using FlowJo.

Antibodies. Rabbit anti-BPV E1 antibodies INA, 1C1, 502-2 in serum (137) and rabbit anti-BPV E2 antibody II-1 (6) were used for ChIP along with monoclonal antibodies to EE and E2 (B201 and B202) in cell culture supernatant. The concentration of these antibodies has not been determined. The amount of each required for ChIP was determined empirically. II-1 requires 5 μl and 502-2 needs 10 μl. Anti-Brd4 rabbit antibody used is AP8051a (Abgent), 5μg/ChIP.

Results

The C127 A3 cell line stably replicates and maintains BPV1. The episomal BPV genome contained within the C127 A3 cells contains three serine-to-alanine mutations in the hinge region of the E2 protein at amino acids 290, 298 and 301. These mutations stabilize E2 and increase genome copy number, as compared with wild type BPV immortalized cells (90, 91). This appears to be due to lack of phosphorylation, mainly on serine 301. This virus also fails to produce the E2R form of the E2 protein which may also result in increased E2 levels and increased viral copy numbers due to lack of repression.

In order to initiate the ChIP studies in C127 A3 cells it was first necessary to test the efficacy of the available antibodies to the BPV1 proteins. A rabbit antibody to E2 (II-1) was raised in this lab and was used in detection of the protein from ID13 cells, the A3 cell line, or the parental C127 cell line. As shown in figure 1.2, the E2 protein was immunoprecipitated from both BPV expressing cell lines using B201 mouse monoclonal anti-E2 antibody with detection by the rabbit antibody II-1. It is interesting to note that the C-terminal repressor form of E2 (E2R) was not detected in the A3 cell line.

A band representing BPV E1 was readily visible in both the A3 and ID13 synchronized cells but not the parental C127 cell line (figure 1.3) after IP and immuno-blot with the 502-2 rabbit antibody. The E1 protein is expressed at very low levels in *in vivo* systems and historically has been difficult to detect.

Figure 1.2. E2 Immunoprecipitation in A3 Cells.
A3, ID13 and C127 cells were immunoprecipitated with the mouse monoclonal antibody B201 with western blot performed using rabbit antibody II-1. The top arrow indicates the band for E2, the bottom the repressor form E2R. Samples on the right contain 5% input.

IP with B201

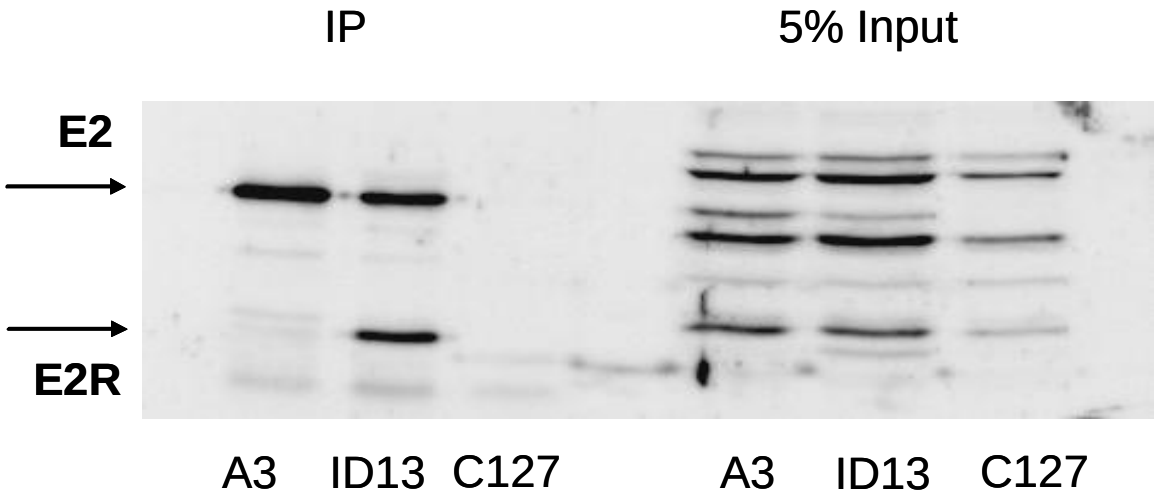


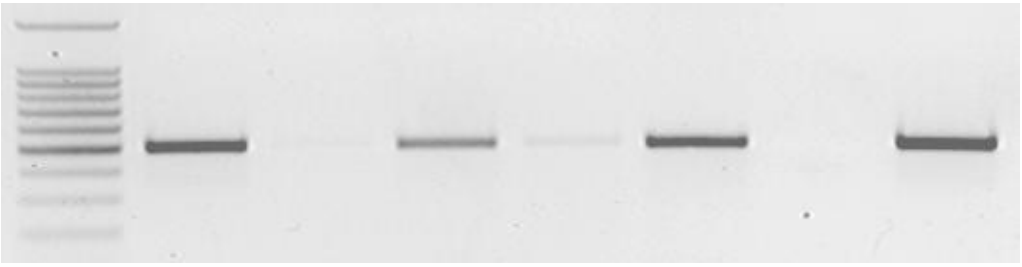
Figure 1.3. E1 Immunoprecipitation in A3 Cells.
BPV1 E1 was immunoprecipitated from A3, ID13 or the C127 parental cell line with the 502-2 rabbit antibody. The immunoblot was performed using a 1:500 dilution of 502-2 and developed using the Thermo-Pierce Dura kit. 64 indicates the location and size of the 64 kDa band in the marker used (See Blue II-Invitrogen).


IP
5% Input
E1
IP with 502-2
64
A3
ID13
C127
A3
ID13
C127

Utilizing the antibodies for both BPV1 E1 and E2, we undertook to ChIP a segment of PV DNA encompassing the viral origin and several of the surrounding E2 binding sites. Pre-immune rabbit serum was used as a negative control for the rabbit II-1 ChIP, and antibody against the EE epitope used as a negative control for ChIP with the monoclonal E2 antibody B202. Both the E1 and E2 ChIPs were clearly positive for BPV origin DNA after 25 cycles of standard PCR using primers located near E2 binding site number 5 within the long control region and downstream of the BPV origin (Primer set C) (Figure 1.4).

The BPV1 E1 protein is expressed at low levels in cells that stably replicate viral episomes. Using asynchronous A3 cells, ChIP was performed using anti-BPV1 E1 antibodies developed in this lab (Figure 1.5) Antibodies were used to detect E1 on the viral genome, utilizing II-1 Ab for E2 as a positive control compared to rabbit pre-immune serum and EE antibody as negative controls. The N-terminal peptide antibody 1NA, and two C-terminal peptide antibodies 1C1 and 502-2, co-precipitated the BPV origin in asynchronous cells using standard PCR with primer set C. This places E1 adjacent to the viral origin at the initiation of BPV replication *in vivo.*

Figure 1.4 E2 Chromatin Immuprecipitation in A3 Cells.
The BPV1 LCR region was isolated in asynchronous A3 cells using chromatin immunoprecipitation. Lane 2-E2 ChIP with rabbit serum antibody II-1. Lane 3-Rabbit pre-immune serum. Lane 4-ChIP with E2 mouse monoclonal antibody B202 supernatant. Lane 5- EE monoclonal antibody ChIP. Lane 6-1% Input. Primer set C for the viral LCR was used to amplify ChIP DNA. The molecular weight marker is a 100 bp ladder in Lane 1 and contains a darker band at 500 bp. Lane 7-Negative control for the PCR contains no DNA. Lane 8-Positive control is the BPV1 genome purified from *E. Coli* DH5α.



1 2 3 4 5 6 7 8

Figure 1.5 E1 Chromatin Immunoprecipitation in A3 Cells.
BPV1 LCR is identified in a chromatin immunoprecipitation assay using IP with anti-E1 antibodies and PCR using primer set C for the viral LCR. Lane3-1NA Rabbit antiserum; Lane 4- 1C1 rabbit antiserum; and Lane 5-502-2 rabbit antiserum. The positive control used is Lane2-E2 II-1 rabbit; and the negative controls include both Lane 7- rabbit pre-immune serum; Lane 8- the anti EE monoclonal antibody. Lane 9- is PCR negative without DNA; Lane 10-The positive control is bacterially expressed papillomavirus genome. A 100 bp marker is included in Lane 1 with the darkest band at 500 bases.

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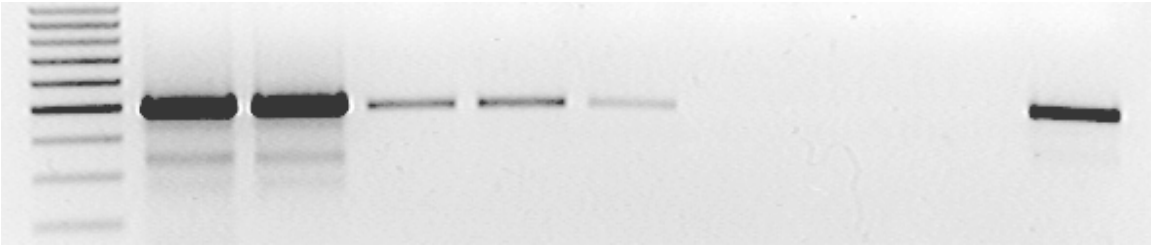


1 2 3 4 5 6 7 8 9 10

To determine whether both E1 and E2 were associated with the viral genome in cycled cells, A3 cells were chemically treated to prevent progression through the cell cycle and allow for synchronization. The cells were effectively blocked in G1/S using 5 mM thymidine (16 hours) or G2/M with 100 ng/ml nocodazole (12 hours). As shown in Figure 1.6A, both the thymidine and nocodazole blocks resulted in well defined peaks when examined using flow cytometry, while sub-confluent asynchronous cells were distributed throughout the cell cycle, with the majority in G1. ChIPs performed on these synchronized cells illustrated the ability to detect E2 in G1/S, ansynchronous cells (Figure 1.4) and cells blocked at G2/M (Figure 1.6B). In contrast, E1 only associated with viral genomes in asynchronous or G1/S blocked cells, but not in cells blocked in mitosis. Figure 1.6.C. shows that E1 is not detectable in mitotic A3 cells.

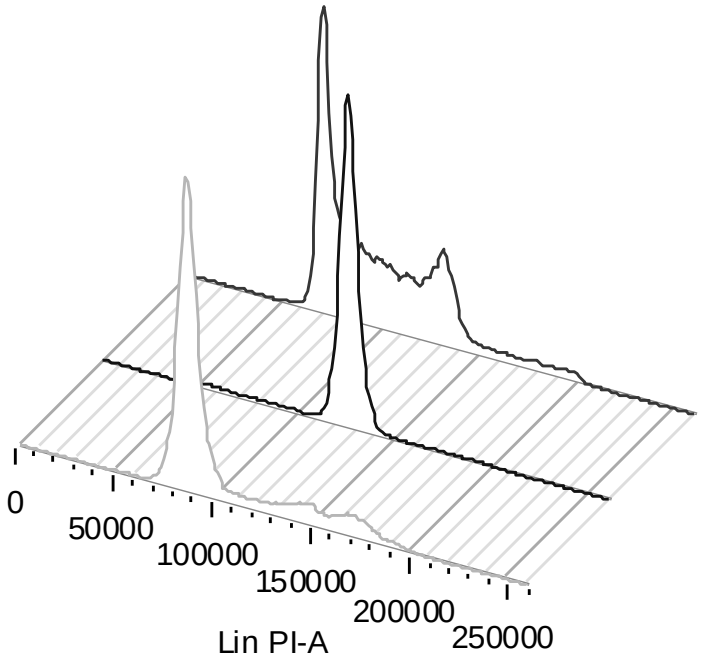
The binding site usage of E2 during the cell cycle in A3 cells was also assessed. The majority of E2 binding sites in the BPV genome are located in the LCR with some as few as 17 bases apart, as illustrated in Figure 1.7. In order to ensure interruption of the viral genome specifically between these LCR binding sites a restriction enzyme digest was added to the ChIP protocol. The BPV1 LCR was examined for restriction enzyme digestion sites that would specifically release the origin of replication from upstream E2 binding sites. Sites for *BanII* and *BglI* were chosen for ChIP due to optimal location in both the LCR and entire genome (Figures 1.7 and 1.10).

Figure 1.6.A. Flow cytometry of A3 cells blocked in the cell cycle. The foremost graph are cells synchronized in G1/S using 5mM thymidine. Cells in the center graph are blocked at pro-metaphase using 100ng/ml nocodazole. The back line depicts asynchronous A3 cells.

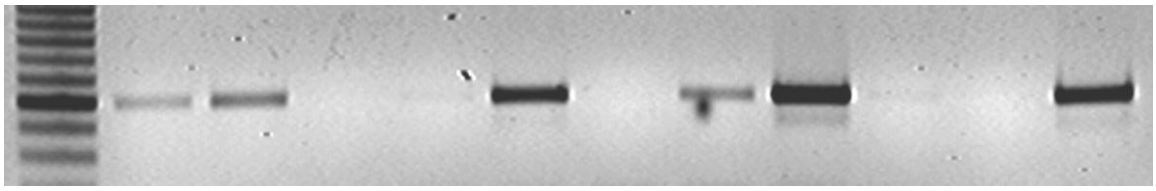
Figure 1.6.B A3 cells are synchronized as described above and submitted for ChIP assay using primers for the viral LCR (primer set C). Lane 1-100 bp marker; Lane 2-E1 in A3 cells blocked in G1/S; Lane 3-E2 G1/S; Lane 4-pre-immune serum in G1/S; Lane 5-E1 in G2/M blocked cells; Lane 6-E2 in G2/M cells; Lane 7-pre-immune serum in G2/M; Lane 8-E1 in asynchronous cells; Lane 9-E2 in asynchronous cells; Lane 10-pre-immune serum in asynchronous cells; Lane 11-negative PCR control; Lane 12-bacterially purified DNA. The 502-2 antibody was used for detecting E1; II-1 for E2.

Figure 1.6.C. C127 or A3 cells were synchronized as described and harvested for immunoblot of BPV-1 E1 using antibody 502-2. Lane 1-C127 blocked in G1/S; Lane 2-A3 G1/S; Lane 3-A3 asynchronous; Lane 4-A3 G2/M. A line depicts the position of the 64 kDa molecular weight marker.

A.


0
50000
100000
150000
200000
250000
Lin PI-A

B.


1 2 3 4 5 6 7 8 9 10 11 12

C.

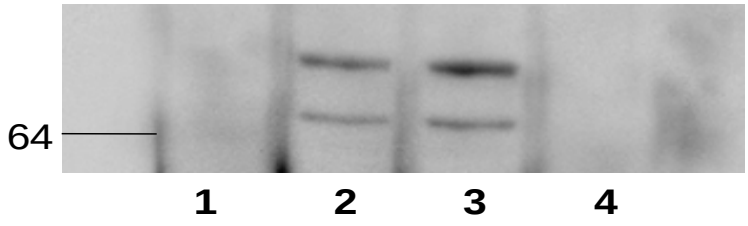

64
1 2 3 4

Figure 1.7 **Nucleotide sequence of the BPV1 LCR**. As shown below E2 binding site are in bold, with the E1 binding site/origin of replication underlined. E2 BS11 and BS12 flank the origin. Several endonuclease digestion sequences are highlighted. This sequence encompasses nucleotides 7140-80.

7141

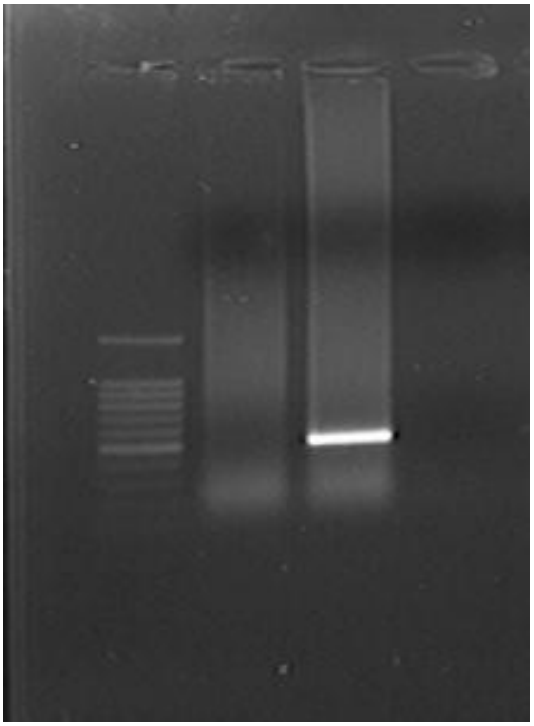
agtcaactgcacctaataaaaatcacttaatagcaatgtgctgtgtcagttgtttattgga**accacaccc**
ggtacacatcctgtccagcatttgcagtgcgtgcattgaattattgtgctggctagacttcatggcgcct
ggcaccgaatcctgccttctcagcgaaaatgaataattgctttgttggcaagaaactaagcatcaatggg
acgcgtgcaaagc**accggcggcggt**agatgcggggtaagtactgaattttaattcg**acctatcccggt**aa
agcgaaagcgacacgctttttttcacacatagcggg**accgaacacgt**tataagtatcgattaggtctat
ttttgtctctctgtcgga**accagaactggt**aaaagtttccattgcgtctgggcttgtctatcattgcgtc
tctatggtttttggaggattagacggggcc**accagtaatggt**gcatagcggatgtctgt**accgccatcgg**
tgc**accgatataggt**ttggggctccccaagggactgctgggatgacagcttcatattatattgaatgggc
gcataatc**agcttaattggt**gaggacaagctacaagttgtaacctgatctccacaaagtacgtt**gccggt**
cggggtcaaaccgtcttcggtgctcgaaaccgccttaaactacagacaggtcccagccaagtaggcggat
caaaacctcaaaaaggcgggagccaatcaaaatgcagcattatattttaagctc**accgaaaccggt**aagt
aaagactatgtatttttcccagtgaataattgttgttaacaataatcac**accatcaccgtt**ttttcaag
cgggaaaaaatagccagctaactataaaaagctgctgacagaccc

80

Bold = E2 binding sites

underlined = E1 binding site Bgl1 site Hpa1 Sau3A1 BanII DpnI BanI

Figure 1.8 Agarose gel of test digest using DNA digested with *Bgl1*. Primer set C was used to detect digested DNA. Lane 1 is 100 bp marker, lane 2 is digested DNA and lane 3 is un-digested DNA. All samples were crosslinked with 1% formaldehyde prior to harvest.



1 2 3

Standard ChIP protocol requires the addition of formaldehyde to a final concentration of 1%. It is possible that the addition of formaldehyde to the cells would crosslink the DNA and compromise the restriction enzyme site. The initial test was to see if digestion could occur under these conditions using cell lysates that were then purified and analyzed by PCR. Figure 1.8 is a test of this principal, and showed that the formaldehyde used did not interfere with the endonuclease digestion.

Three sets of primers were designed to detect ChIP products from this region of the LCR as depicted in figure 1.9 and materials and methods. The first set (A) amplified nucleotides 7867 to 297 surrounding the viral origin. The second set of primers (B) encompasses nucleotides 7205-7685 and amplified DNA upstream of the BPV origin. The third set of primers, primer set C, flanked both the *BanII* and *BglI* restriction sites from nucleotides 7522 to 67. Digestion with *BanII* at nucleotide 7653 and *BglI* at 7832 facilitated the examination of the LCR close to the origin of replication, specifically allowing for detection of these separated regions by PCR. Digestion at these sites was designed to delineate origin binding (primer set A) from upstream E2 binding sites (primer set B), with primer set C serving as a control for the ChIP and to demonstrate efficient cleavage by the endonucleases, as efficient cleavage at these sites prevented formation of a PCR product using primer set C (See diagram Figure 1.9).

ChIP in combination with restriction enzyme digestion was applied to thymidine blocked A3 cells and binding to the BPV origin was subsequently examined by PCR. The fragment of LCR amplified by primer set C was

precipitated by both E1 and E2 in undigested samples (Figure 1.9.C). Digestion by both *BanII* and *BglI* resulted in absence of product amplification, due to the restriction sites dissecting the genome between the PCR primers (nucleotides 7522-67), forming an efficient control for both ChIP and enzyme digestion.

Figure 1.9.B. shows amplification of region B of the LCR upstream of the origin (7205-7685) from the thymidine blocked A3 cells. This region of the DNA contains multiple E2 binding sites (sites 1-10). E1 did not bind this region of the viral genome in either digested or undigested samples. However, E2 was detected at this region in undigested and *BglI* digested samples, but not in those subjected to *BanII* digestion due to the location of the PCR primers which flanked this cut site. Nucleotides 7867-297 span BPV binding sites 11 and 12 as well as the BPV E1 origin of replication (Figure 1.9.A.). PCR with primer set A detects both E1 and E2 bound to the genome at the origin of replication in cells blocked in G1/S. The results from this panel of PCR primers indicate that, in S phase, E2 binds to several sites in the viral LCR, binding upstream and remaining at the origin of replication.

Panels D and E of Figure 1.9 demonstrate results obtained from ChIP of the LCR region in cells synchronized at early mitosis with nocodazole. E2, and not E1, immunoprecipitates the genome in these cells. Binding to the E2 cluster region of the LCR surrounding the MME, evidenced by a band in the *BglI* lane of 1.9.E, is faint, but may be indicative of specificity in this region. Binding is evident in the LCR (1.9.D).

To acquire a more complete picture of the association of E2 with the genome in cycling cells additional ChIP/restriction enzyme digestions were performed. The restriction enzyme *PflM1* (nucleotide 3682) was used in combination with *BglI* (nucleotides 618, 2818, 6531 and 7832) resulting in the absence of E2 binding sites in connection with the L1 coding sequence (See Figure 1.10).

Figure 1.9. E1 and E2 on the Genome in Cycled, Digested A3 Cells. The cartoon on the right depicts the primer scheme used to determine where E1 and E2 immunoprecipitated on the viral genome in thymidine blocked cells following digestion with either *Bgl1* or *BanII*. Panel A was amplified using primer set A flanking the viral origin. Panel B utilized primer set B to identify DNA precipitate from the LCR regions upstream of the origin and panel C encompassed both restriction enzyme sites and the origin as well as upstream E2 binding sites.

Panels D and E are ChIP of nocodazole blocked cells with PCR for the upstream E2 cluster (D) or the entire LCR (E). Letters on the right of diagram indicate primers used.

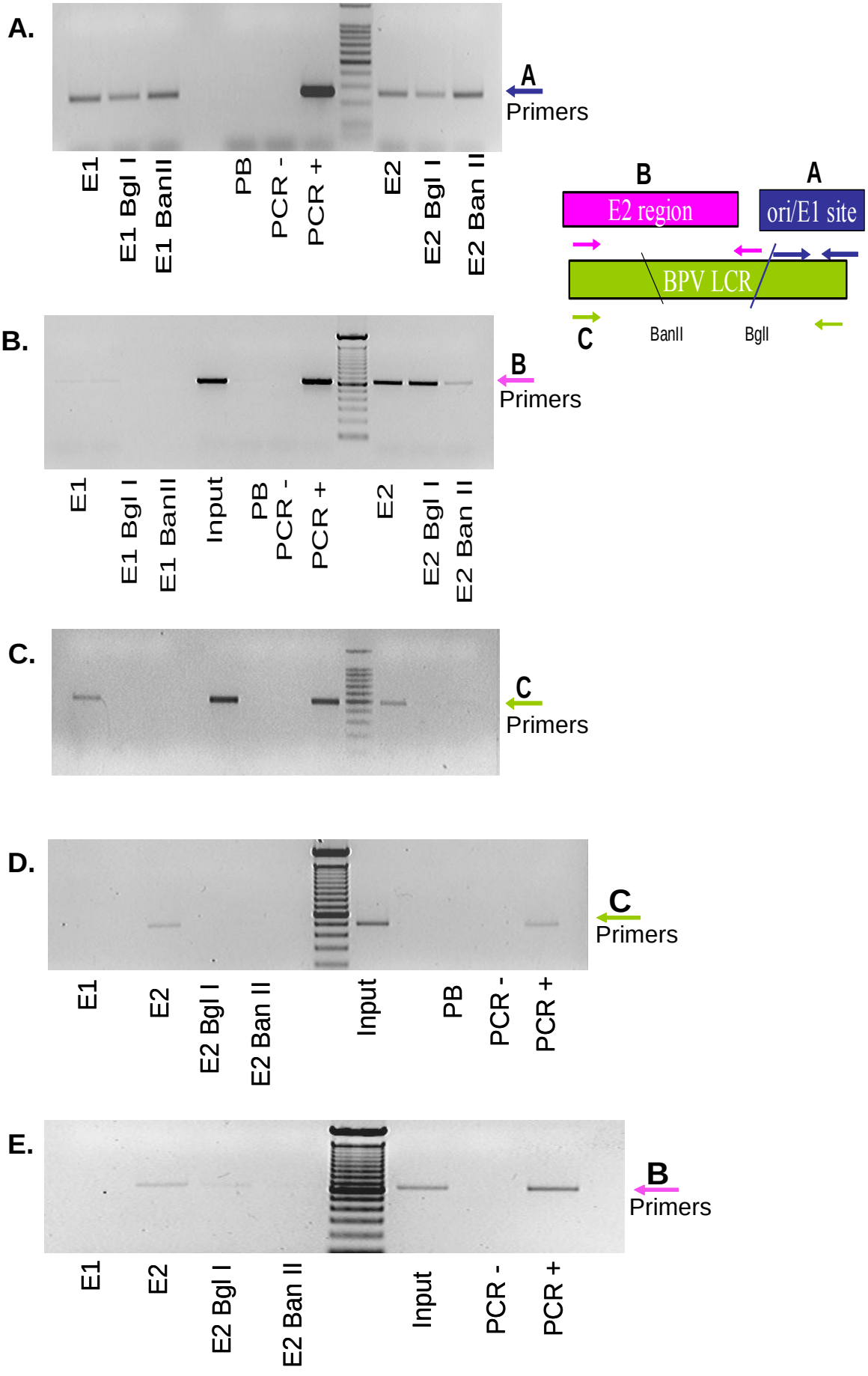
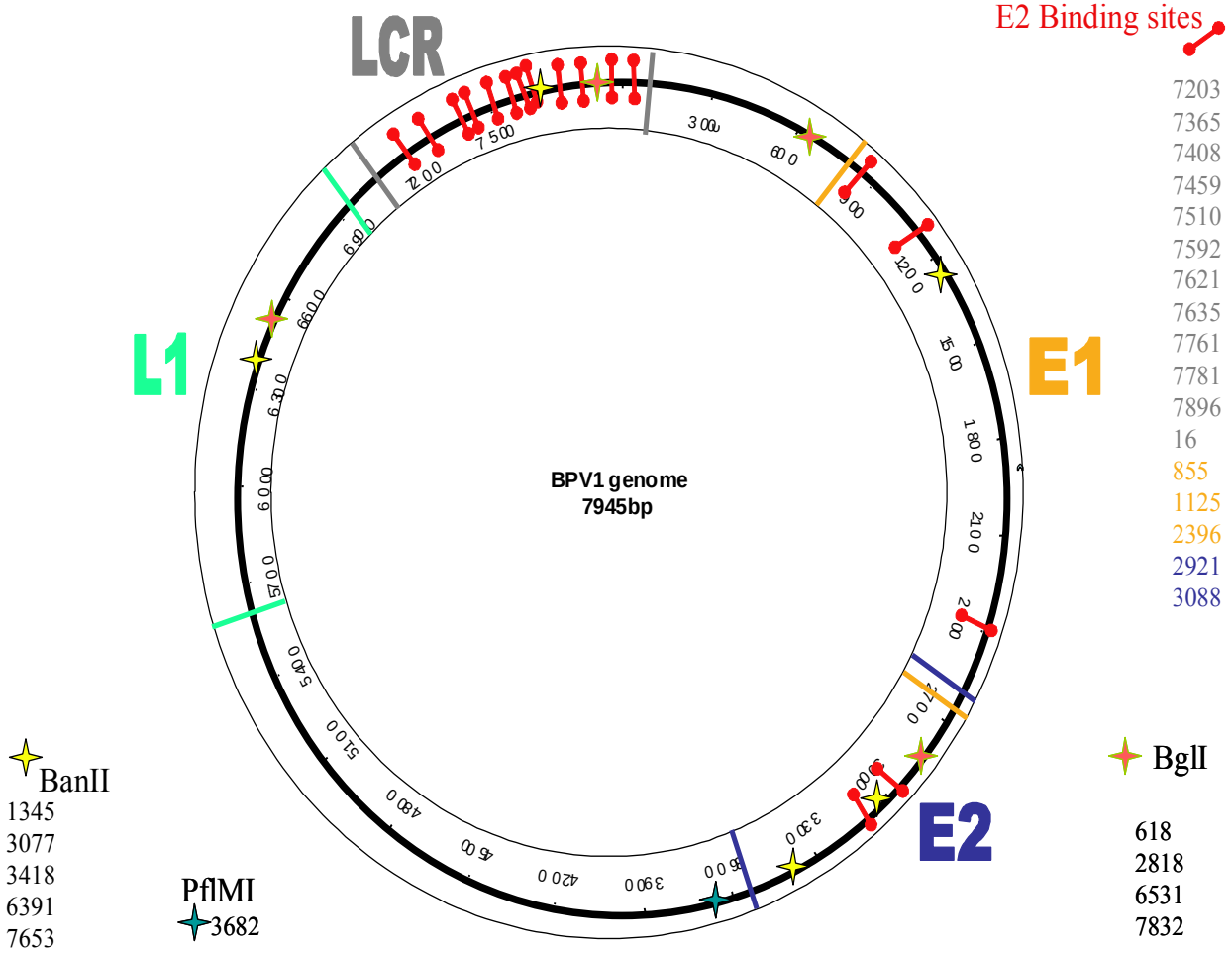

A.
A
Primers
E1
E1 Bgl I
E1 BanII
PB
PCR -
PCR +
E2
E2 Bgl I
E2 Ban II
B
A
E2 region
ori/E1 site
BPV LCR
C
BanII
BglI
B.
B
Primers
E1
E1 Bgl I
E1 BanII
Input
PB
PCR -
PCR +
E2
E2 Bgl I
E2 Ban II
C.
C
Primers
D.
C
Primers
E1
E2
E2 Bgl I
E2 Ban II
Input
PB
PCR -
PCR +
E.
B
Primers
E1
E2
E2 Bgl I
E2 Ban II
Input
PCR -
PCR +

Figure 1.10 Restriction Enzyme Sites in the BPV1 Genome.
Schematic of BPV1 genome including E2 binding sites *BanII*, *BglI*, and *PflMI* sites.


LCR
L1
E1
E2
BPV1 genome
7945bp
E2 Binding sites
7203
7365
7408
7459
7510
7592
7621
7635
7761
7781
7896
16
855
1125
2396
2921
3088
BanII
1345
3077
3418
6391
7653
PflMI
3682
BglI
618
2818
6531
7832

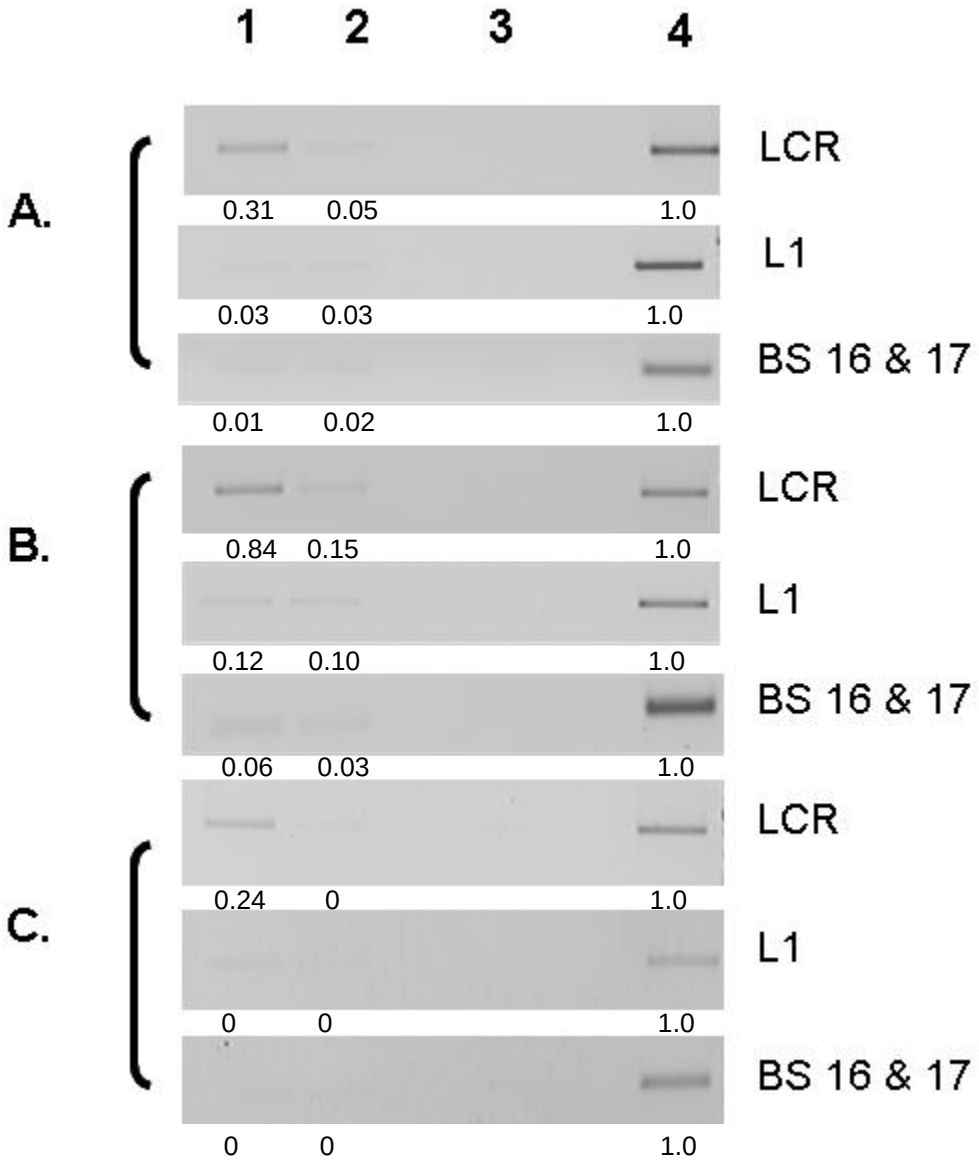
The LCR was amplified using primer set C in all undigested PCR reactions (Figure 1.11 lane 1, LCR), while the restriction enzyme controls using *BglI* digestion and LCR primers were all negative (lane 2 LCR). This demonstrated that the viral DNA was efficiently digested, at least in the LCR region. Nucleotides 5638-6039 were amplified with primer set L1 (Figure 1.11, L1). The E2 antibody did not ChIP this region in digested or undigested samples (Figure 1.11, lane 1) consistent with the absence of E2 binding sites (Figure 1.11, lane 2, L1).

The sequence between the *BglI* restriction site at 2818 and the *PflM1* site at 3682 contains E2 binding sites 16 and 17. This region was amplified from the ChIP DNA samples using primers for nucleotides 2953-3085; however, the E2 binding was absent in both undigested and in digested ChIPs (Figure 1.11, BS 16&17). Only faint binding was detected at any of the distal sites outside the LCR in any phase of the cell cycle, G1/S, asynchronous or mitosis, could be detected during these studies (compare A, B and C groups, Figure 1.11).

Quantification of these PCR results using ImageJ software is shown in Figure 1.11. While this PCR is meant to be qualitative, distinct differences can be observed between the positive E2 binding in the LCR when compared to both L1 and BS16&17. The most significant positive outside the LCR is detectable in asynchronous cells in the L1 region. Binding here is 7 fold lower than that detected in the LCR region of the genome in this sample.

Figure 1.11. Off-origin ChIP of E2 in Cycled A3 Cells.
A3 cells were synchronized and ChIPped for BPV E2 (lane1), then ChIPped for E2 and digested with *BglI* and *PflM1* (lane 2). Lane 3 is undigested ChIP sample using rabbit pre-immune serum, and Input diluted 1:10 is also shown (lane 4). PCR was performed using either primers for the LCR of BPV, for nucleotides 5638-6039 in the L1 region (L1) or for nucleotides 2952-3085 flanking E2 binding sites 16 and 17 (BS 16&17). Samples in 1.11.A. are synchronized at G1/S, 1.11.B. are asynchronous and 1.11.C. are blocked at pro-metaphase.

PCR samples were quantified using ImageJ software and the results shown below each panel. Background was sub-tracted then samples were normalized to input. Negative values were set at zero.


1
2
3
4
A.
LCR
0.31
0.05
1.0
L1
0.03
0.03
1.0
BS 16 & 17
0.01
0.02
1.0
B.
LCR
0.84
0.15
1.0
L1
0.12
0.10
1.0
BS 16 & 17
0.06
0.03
1.0
C.
LCR
0.24
0
1.0
L1
0
0
1.0
BS 16 & 17
0
0
1.0

Brd4 has been shown to be responsible for tethering of the viral genome to cellular chromatin during mitosis (190). There is some ChIP data illustrating binding of Brd4 to the viral genome, however it is unclear whether this data was obtained in mitotic cells (190). We performed a ChIP assay on A3 cells to determine the genomic localization of Brd4 in mitotic A3 cells. Figure 1.12 shows a ChIP assay for E2 and Brd4 in cycled A3 cells in the absence of restriction endonuclease digestion. Results showed that Brd4 did not ChIP with the viral genome in either thymidine blocked cells (Figure 1.12.A.) or nocodazole blocked cells (Figure 1.12.C, lane 2). However, association was detected in asynchronous cells (Figure 1.12.B, lane 2), consistent with a role for Brd4 in viral transcriptional regulation.

In order to examine whether both E1 and E2 were bound to the viral origin simultaneously we attempted a re-ChIP of the BPV LCR (Figure 1.13). ChIP samples were washed, eluted from the antibody, then another ChIP was performed using either the same antibody (positive control) a second antibody, or pre-immune serum (negative control).

Figure 1.12. ChIP of Brd4 on the BPV1 genome. Primer set C for the BPV LCR was used. Lane 1 is II-1 E2 Ab; Lane 2 is Brd4; Lane 3 pre-immune serum; Lane 4 is input. The molecular weight marker is a 100 bp ladder, with a darker band at 500 bases. Panel A thymidine blocked (G1/S), Panel B asynchronous, Panel C nocodazole blocked (M).

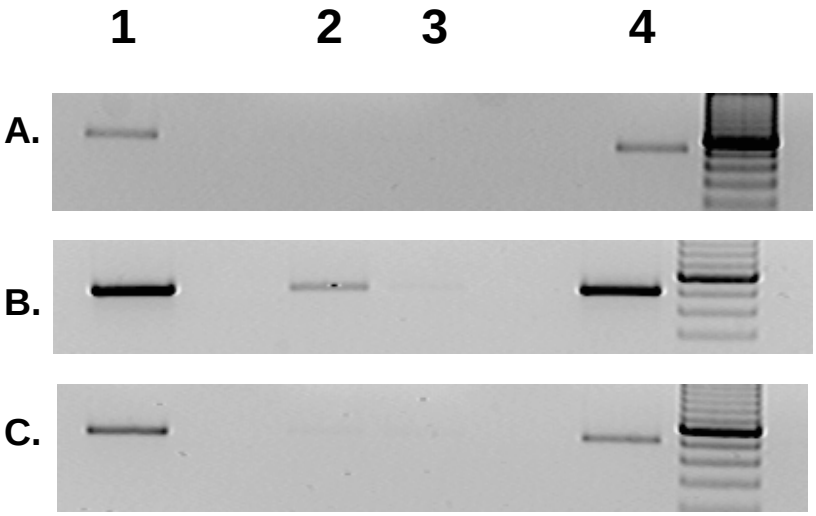
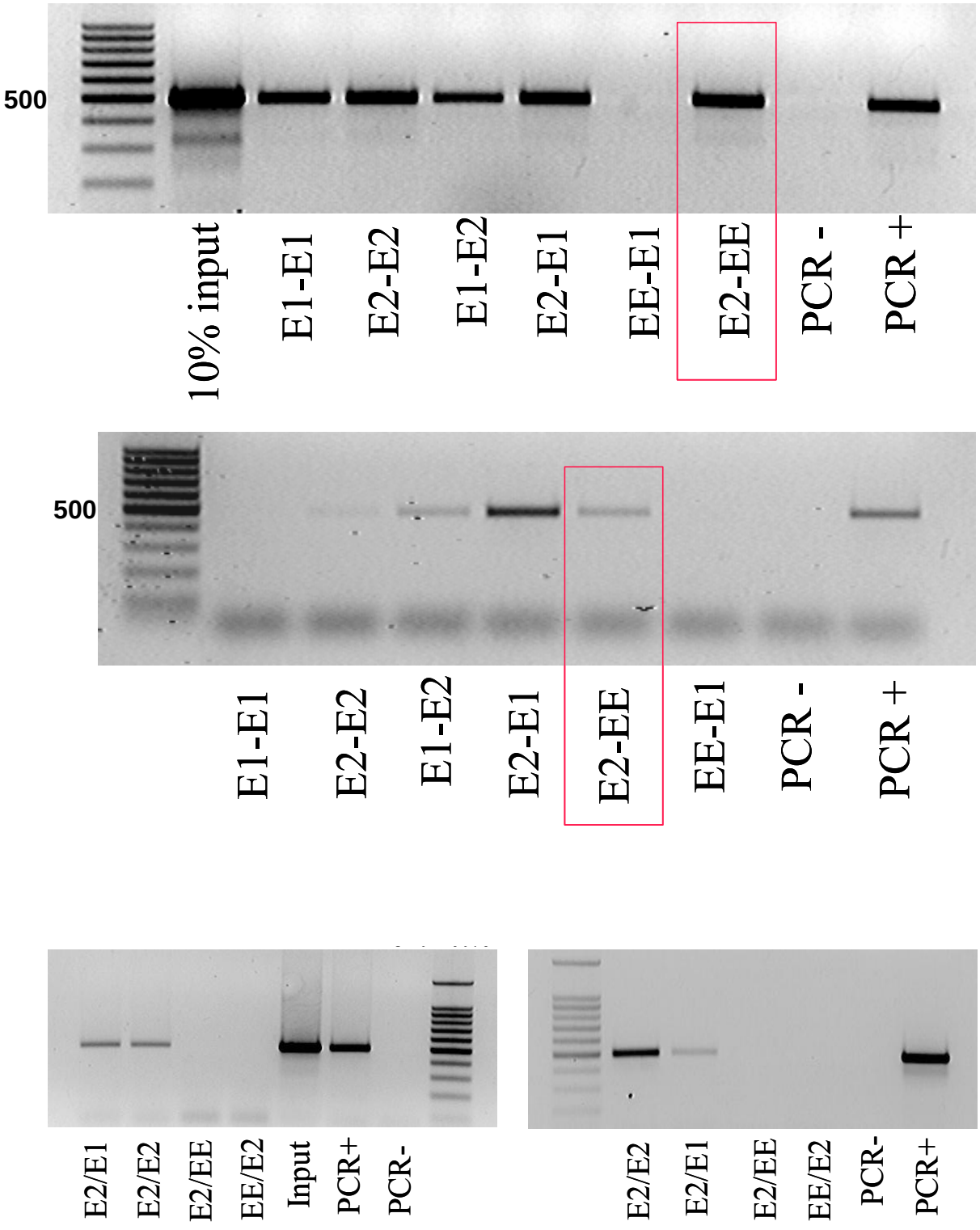

1
2
3
4
A.
B.
C.

Figure 1.13 Re-ChIP

Two separate attempts at a sequential re-ChIP of the LCR. Antibodies are listed below figure in order of use. The E2 antibody II-1; E1 502-2; EE is monoclonal antibody to the EE epitope tag. The molecular weight marker is a 100 bp ladder, with the darkest band at 500 bp.

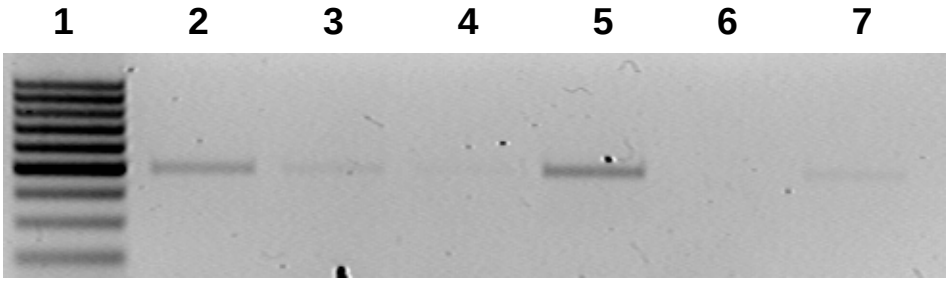
The bottom panels depict two successful re-ChIP experiments in A3 cells stalled at G1/S with 5mM thymidine.


500
10% input
E1-E1
E2-E2
E1-E2
E2-E1
EE-E1
E2-EE
PCR -
PCR +
500
E1-E1
E2-E2
E1-E2
E2-E1
E2-EE
EE-E1
PCR -
PCR +
E2/E1
E2/E2
E2/EE
EE/E2
Input
PCR+
PCR-
E2/E2
E2/E1
E2/EE
EE/E2
PCR-
PCR+

High background occurred in all lanes in which E2 was used as the primary antibody. Additional ChIPs were performed in thymidine blocked cells to see if the background could be cleared using additional high salt washes. Two ChIPs showed minor background, with clean positives, but the results could not be repeated (Figure 1.13 bottom panel). These samples are undigested ChIPs, and as such show only that E1 and E2 are on the same larger DNA fragment. Digestion would be required to determine whether these proteins are bound together near the origin.

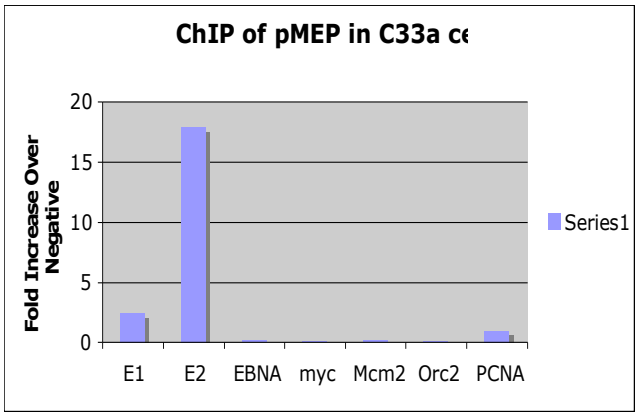
In an attempt to reduce background the primary and secondary antibodies were both cross-linked to the Sepharose beads using dimethyl pimelinidate dihydrochloride (Figure 1.14). No improvement was seen as a result of this additional step.

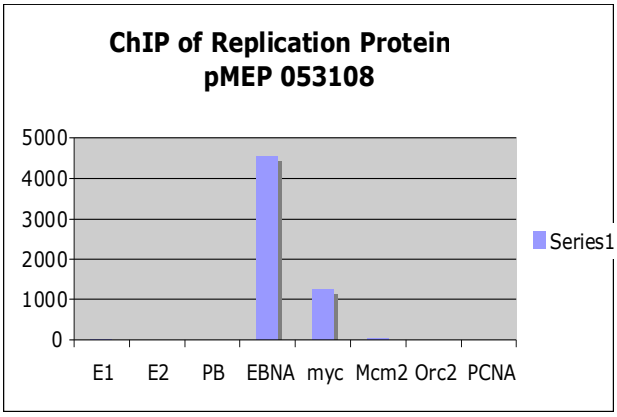
Figure 1.14 Cross-linked re-ChIP.
A re-ChIP with antibodies that had been cross-linked to protein A Sepharose beads prior to immunoprecipitation. E2 anitbody used was II-1, E1 antibody 502-2. Lane 1- 100 bp marker with emphasis beginning at 500 bp; Lane 2-E2/E2; Lane 3-E2/E1; Lane 4-E2/pre-immune serum; Lane 5- pre-immune serum/E2; Lane 6-PCR(-) no template; Lane 7-PCR (+) plasmid DNA.


1
2
3
4
5
6
7

The ChIP assay was effective in the A3 cell line with the endogenous viral proteins E1 and E2. We thought to broaden the study to examine cellular factors that might be present at the viral origin and required for BPV1 DNA replication. An EBV containing plasmid, pMEP was the positive control for these experiments. In order to compare both viral origins, the A3 system was abandoned in favor of transfection into the C33a cell line. The main advantage to working with C33a is that these cells are an adapted HPV negative cervical cancer line, allowing for use of antibodies to human replication proteins. The main drawback is that it is not known whether the BPV1 genome will replicate in this cell line. Primers were constructed for real time PCR (RT) on both the pMEP plasmid and the BPV1 genome. One Φg of each was transfected into a 10cm dish of C33a and cells were synchronized with 5 mM thymidine at 24 hours post transfection. Cells were harvested at 36 hours and ChIP assay performed using 5-10 Φg of antibody. Quantitative RT was performed on the samples (Figure 1.15). ChIP on the transfected cell line did not give reliable results using quantitative PCR. The same samples used for two real-time assays showed remarkably different results when the annealing temperature for the PCR was changed from 58E (left panel) to 60EC (right panel). Results were calculated using fold increase over negative control, or arbitrary units of DNA.

Figure 1.15 ChIP of Replication Proteins in C33a Cells.
The graph on the left is RT of C33a cells transfected with pMEP and immunoprecipitated. The antibody is labeled on the X-axis. Y-axis shows fold increase over negative control. The right graph depicts the same samples with increased annealing temperature of 60 vs 58, and with arbitrary units of DNA on Y-axis.


ChIP of pMEP in C33a ce
Fold Increase Over Negative
20
15
10
5
0
E1
E2
EBNA
myc
Mcm2
Orc2
PCNA
Series1


ChIP of Replication Protein
pMEP 053108
5000
4000
3000
2000
1000
0
E1
E2
PB
EBNA
myc
Mcm2
Orc2
PCNA
Series1

Discussion

The results placing the E1 protein at the site of initiation of viral replication on the complete genome in a cell culture system are not unique. Nonetheless, along with the immunoblot data, the figures are interesting in that the levels of E1 produced from the viral genome are so low that the protein is not normally detectable. Here E1 was evident in both the A3 cell line and the ID13, and the ChIP results were measurable and repeatable. We also show that E2 remains associated with the genome, regardless of the phase of cell cycle. While extensive work has been undertaken to characterize the E2 binding sites within the genome of the bovine papillomaviruses, the majority of these studies utilized either *in vitro* systems or *in vivo* models where E1 and E2 or portions of the viral LCR were transfected into cell lines (21, 72, 95, 100, 111, 131). Ustav *et al*. used mutated E2 binding sites, including BS12, and cell lines stably expressing both E1 and E2 to examine the requirement for E2 at the origin of replication (170). Sedman and Stenland use EMSA and DNAse I footprinting of E1 and E2 expressed from *E.coli* to examine the binding of E1 and E2 to the viral origin (146). Lusky *et al.* (100) and Yang *et al.* (186) developed cell free systems which utilized portions of the LCR combined with E1 alone (100) or E1 and E2 (186) and examined the effects of mutations and variations and expression levels of E1 and E2. Piirsoo *et al* used portions of the viral genome containing, or lacking, specific E2 binding sites and partially characterized E2 mutants to determine the *cis* requirements for the MME in stable maintenance (131). These *in vivo* and *in*

vitro models utilized E1 and E2 at levels notably higher than endogenously expressed protein and do not allow for the influence of the cell cycle on viral protein levels, expression, modification or nuclear localization.

In comparison to the above, the method adapted by this lab utilizes the C127 A3 cell line that stably replicates the entire BPV1 genome with serine to alanine mutations in the E2 protein at amino acids 290, 298 and 301. In wild type BPV1, phosphorylation, ubiquitination and degradation of E2 seem to rely heavily on phosphorylation at serine 301, and mutation of this serine alone results in raised levels of E2 which increases the availability of E2 for tethering during mitosis and enhances viral DNA copy number (91, 129). This cell line also lacks the E2R repressor element often found in replicating wild type BPV1 systems (Figure 1)(86). This may lead to increased viral replication over wild-type BPV1 systems. The use of a stably replicating cell line, despite the mutations, allows for formation of nucleosomes on the viral genome and regulation of the viral gene products in a manner more consistent with *in vivo* systems when compared with previous studies.

Traditional ChIP has been used to distinguish between closely aligned transcription factor binding sites within cellular genomic DNA utilizing only sonication to disrupt the chromatin. For our purposes sonication resulted in variable sized fragments with no consistent restriction points and in the majority of cases sonication alone was unable to disrupt the genome between the tightly packed E2 binding sites of the LCR. This may be due to the size constraints of the BPV genome, or alternatively the traditional ChIP may have insufficient

resolution for differentiating between close DNA binding sites and subsequently may be more useful when examining promoters or other DNA elements separated by greater distances. At a lower concentration of formaldehyde (.33%) sonication alone was able to separate the origin from the genome to some extent (Figure 1.9.B.), but proved to be inconsistent. Therefore, the combination of traditional ChIP with restriction enzyme digestion ensured the specificity of the DNA binding by E2. BPV1 E1 binds specifically to the viral origin and can be immunoprecipitated on this site in genomes initiating replication. E1 can therefore serve as a positive control in future ChIP assays to examine cellular factors required for BPV1 replication.

While the A3 cell line performed relatively well when synchronized, there were minor limitations imposed on this study by the irregularity of cycling. Serum starvation did not maintain cells in G1, as a subpopulation continued to cycle (data not shown). Thymidine blocks cells at the initiation of replication by inhibiting the synthesis of deoxycytidine and initiating the DNA repair response. This stalls the progression of replication forks and delays the transition into S phase (13, 181) and was employed here to analyze cells that were blocked at the border of G1/S at the initiation of viral replication. Our intent was to trap E1 and E2 at the onset of replication. A double thymidine block is the more common approach, but a double block resulted in a significant amount of cell death with the A3 cell line. As shown in Figure 1.6.A. a single thymidine block resulted in the majority of cells blocked at G1/S. Also, a significant number of the cells released from a single thymidine block would not progress into S phase along with the rest

of the population, despite repeated washing, resulting in asynchrony. This made it impossible to collect data resulting from direct release from serum starvation or thymidine block and subsequent progression through the cell cycle, necessitating the use of nocodazole for examining cells blocked in mitosis. Nocodazole acts as a microtubule depolymerizing agent and prevents assembly of the mitotic spindle required for genome segregation, effectively blocking cells in prometaphase. This block does not interfere with BPV1 E2 mediated segregation of episomes, which is independent of mitotic spindle formation (30), nor should it interfere with Brd4 mediated attachment to chromatin, as Brd4 has been shown to localize to condensed chromatin with only minor staining visible on mitotic spindles (1, 190).

Although Brd4 is a potential candidate for the cellular protein required for viral tethering, neither our lab nor Ives *et al* were able to place Brd4 on the viral genome in mitotic cells using chromatin immunoprecipitation (71). It is possible that the association of Brd4 with E2 and the viral genome prior to mitosis is the key to its role in tethering, or it is also possible that the assays employed are not sensitive enough to detect the interaction of this protein with the genome during prometaphase. Our data is consistent with the published role for Brd4 in mediating viral transcription (71, 89, 105, 145, 179).

Our data does show that E2 remains associated with the BPV LCR throughout the cell cycle, even appearing on the viral MME at G1/S in addition to binding near the origin of replication. It has been reported that central LCR E2 binding sites, predominantly sites 5-8, are the *cis* element required for BPV1

genome tethering (131). Our results confirm that the E2 protein is specifically bound to the LCR of the viral genome in cells blocked at the onset of mitosis.

BPV1 E2 binding sites 11 and 12 are found flanking the viral origin of replication. It has been reported that the binding of E1 to the viral origin excludes E2 from BS12 (100). Our data does not differentiate between binding to BS11 or to BS12 in cells blocked at G1/S (Figure 1.9); neither does it verify that E1 is present on the origin while E2 is bound to BS12. The use of sequential ChIP of the origin would be necessary to show concurrent association of both viral proteins. Attempts at this technique were not effective due to high background, but initial data is promising. Additional restriction enzyme digestion would also be required to differentiate between binding of E2 to sites 11 and 12, both of which have been shown to be capable of a role in initiating replication *in vitro* (136).

The BPV E2 binding sites have been determined based on both sequence similarity and detection of E2 bound to DNA *in vitro* using electrophoretic mobility shift assay (EMSA). The affinity of several of these binding sites is remarkably low, leading us to question their functionality. The binding sites outside the viral LCR do not appear to be required for growth or maintenance of the monolayer cultured transformed cells. However, it is possible that they are utilized during growth of differentiated cells. It is also interesting that the inability to detect the E2R mutant in A3 cells correlates with the absence of E2 at the putative E2R promoter, which is thought to regulate its expression. Why the A3 mutant would not bind to this site *in vivo* is uncertain.

The herpesvirus protein Epstein Barr Nuclear Antigen 1 (EBNA-1) is similar in function to E2. It too is multifunctional with roles in replication, transcription and viral maintenance. The DNA binding and dimerization domain of EBNA-1 folds in a beta-barrel structure similar to E2 (reviewed in (54, 97)) and binds to the cellular protein Brd4 during mitosis (96). The genome of EBV however is more than 20 times the size of the BPV genome and in contrast to the PV genome, which may replicate several times per S phase, replicates only once per cell cycle being subject to the restrictions of DNA licensing factors in the cell (97). While ChIP assays have placed a number of cellular factors at the EBV origin (18), our lab is currently using this technique to compare these systems and determine the cellular factors required for PV replication in both monolayer and differentiated keratinocyte culture systems. The C33a system has shown to be undesirable for determining origin binding of BPV because the positive and negative controls were not reliable and additional work would be required for optimization. Instead of repeating EBNA results previously published, a negative control antibody will be used and E1/E2 will serve as reliable positive controls.

There are several technical points to keep in mind when performing the ChIP and restriction enzyme digest (RED-ChIP) assay. Formaldehyde oxidizes quickly and should be used within 30 days of opening. Precious time was wasted attempting to get the assays back on track after using old formaldehyde. Assuming fresh formaldehyde, only .33% final volume is required for RED-ChIP cross-linking as compared to the traditional ChIP. The restriction enzymes need to be checked periodically as well. Incomplete digestion was also another factor

in wasted experiments, especially with highly concentrated enzymes, which seem to become inactive more quickly than those of lower concentration.

The use of ChIP combined with the restriction enzyme digest is a useful tool for finer mapping of transcription start sites in regions of the genome where these sites are within several nucleotides of each other with a restriction enzyme site between. It may be possible, utilizing this technique, to analyze the binding of E2 to its sites in the genome in differentiated human keratinocytes containing the viral genome to determine the occupancy of E2 binding sites throughout the viral replicative program.

Preface

Work in this section was supplemented by Joanna L. Parish PhD and Angela M. Bean, a current graduate student at the University of Massachusetts Graduate School of Biomedical Sciences. Portions of this work have been submitted for publication and are currently under revision.

Association of Cohesin with the papillomavirus E2 protein and episomal viral genomes. Joanna L. Parish, Suzanne M. Melanson, Angela M. Bean and Elliot J. Androphy. Submitted J. Virol.

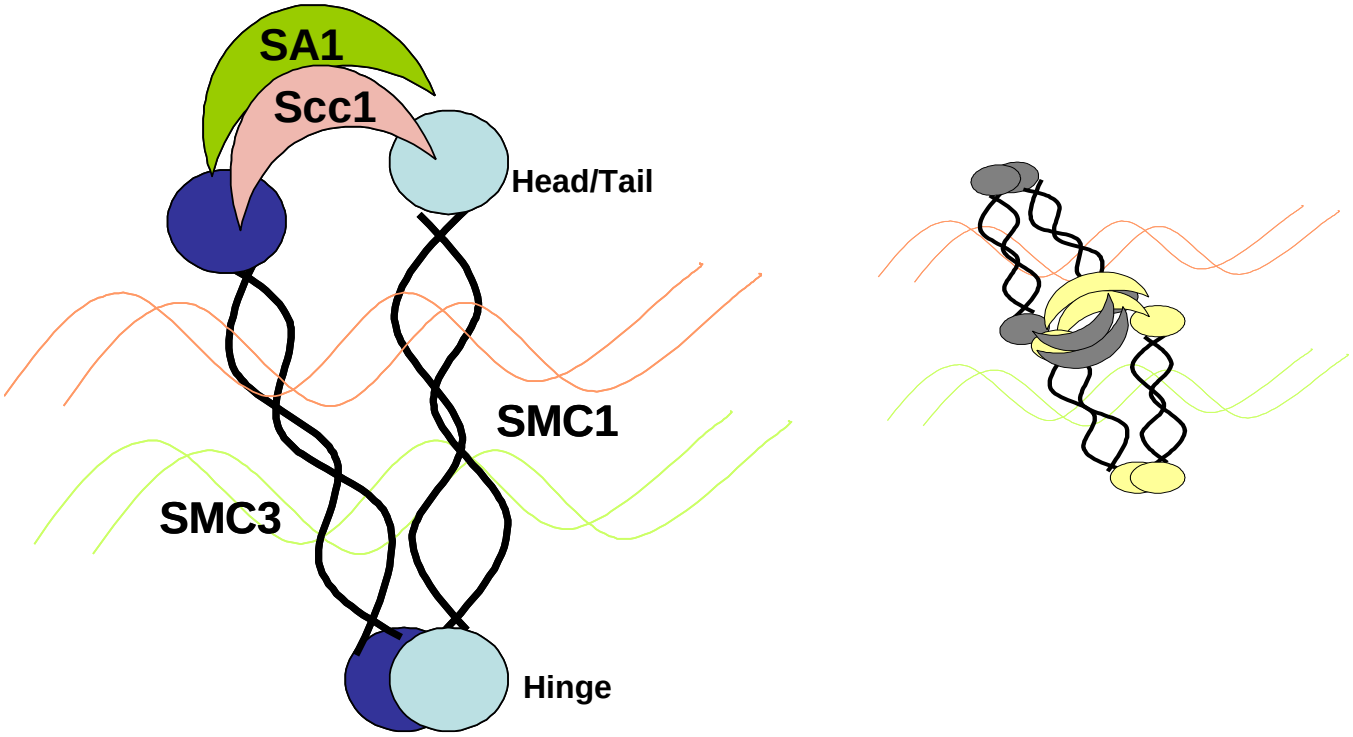
Chapter II:

E2 and the Cohesin Protein Scc1

The Cohesin Proteins

Upon completion of S phase, each cell contains two copies of its genetic information in the form of sets of sister chromatids. Cohesion, which is established during replication, ties newly replicated sister chromatids together until the onset of anaphase, where dissolution of the ties that bind allows for complete and perfect segregation of these chromatids into the newly forming daughter cells. Correct and timely segregation of sister chromatids is essential for maintaining the eukaryotic genome. As mitosis approaches, the pairs of sister chromatids align along the metaphase plate. The kinetechores of the sisters attach to opposite centrosomes through the mitotic spindles. Only when each chromatid is aligned, and applying tension to its attached spindle, are the two able to separate through the dissolution of cohesion (Reviewed (119)). Two methods of cohesion have been postulated, and it is possible that both are required for the proper maintenance of genome copy number. The first method proposed was catenation (117). Catenation involves the intertwining of genomes as a result of replication and physically ties the creation of cohesion to S phase of the cell cycle. The second method, and the most widely subscribed to, establishes a biochemical link between S phase and cohesion and this method is the formation of the cohesin complex (57, 109).

Figure 2.1 Cartoon Depiction of Cohesin Structure.
The cartoon depicts the structure of the cohesin complex with the N-C terminal heads of Smc1 and Smc3 binding to Scc1, with their tails forming the hinge region with a coiled coil structure in between. The large figure suggest cohesin engaging sister chromatids together, while the smaller insert suggests an alternate model where each cohesin ring binds one sister, with the cohesion being established by the interaction of the two rings.


SA1
Scc1
Head/Tail
SMC1
SMC3
Hinge

The cohesin complex is formed by the association of the proteins Structural maintenance of Chromosome 1 (Smc1) Smc3, and Scc1 in a trimeric ring-like structure (55). The SA1 protein is also involved, in contact with Scc1, to form a structure large enough to encircle both sister chromatids engaged with histones. As depicted in figure 2.1, the heads of Smc1 and Smc3 are globular domains which function to bind ATP and Scc1. The N-terminus of Scc1 binds the Smc3 head with the C-terminus binding the Smc1 head. The hinge region, or tail, of Smc is required for dimerization of the SMC proteins (58). A flexible anti-parallel coiled-coil domain connects the head and tail, with the N and C termini both comprising the globular domain which encompasses the ATP binding domain (106). Although cohesin has been shown to be large enough to encompass both sister chromatids, as depicted in figure 2.1, an alternate hypothesis suggests that each cohesin ring encircles only a single sister, and that it is actually the two rings which intertwine to keep the strands together (see small insert 2.1) (191).

The exact mechanism of establishment of cohesion and the complete role of the cohesin complex have yet to be fully elucidated. Several models exist for the deposition of cohesin onto DNA and for its transmission to newly replicated chromatin (reviewed (152)). It was originally postulated that cohesion was established during replication (168). In mammalian cells, cohesin has been shown to bind DNA as early as telophase in preparation for the next round of replication where the proteins Scc2 and Scc4 are required for loading of the cohesin complex (23). Loading may occur in a step-wise manner (151) or by the addition of pre-formed complexes directly onto the DNA followed by DNA

replication through the center of the cohesin ring (23, 98). However, it has also been shown that the fully formed cohesin complex exists free of chromatin and can associate with DNA without a requirement for replication (168).

New evidence in a mammalian system suggests that cohesin binds to DNA dynamically beginning in telophase through G1, becomes stably bound throughout S phase peaking at the onset of G2, and the amount stably bound decreases again through prometa-metaphase to be dissociated at anaphase (46). This would reinforce the theory that actual establishment of cohesion through the cohesin complex requires S phase replication of DNA, still allowing for the association of the cohesin complex and accomplishment of tasks not related to cohesion throughout interphase through transient binding.

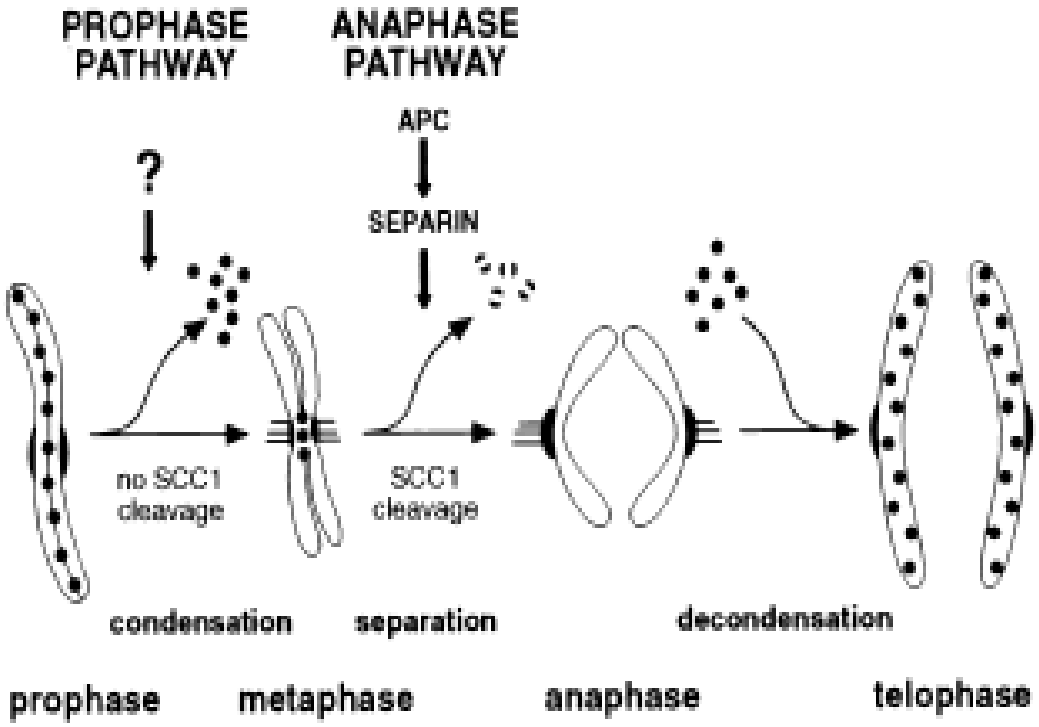
The Scc1/Smc1/Smc3 complex has been shown to be recruited to sites of DNA damage and to insulator regions of both cellular and viral DNA. The cohesin complex is recruited to the site of double strand breaks (DSB) in mammalian cells by the Smc5 and Smc6 proteins (132). Once localized to the site Smc1/3 are phosphorylated by the kinases ATM (ataxia telangiectasia mutated) and ATR (78, 189). The exact role of cohesin in DSB repair is not known. It has been shown, however, that in response to DNA damage cohesin can be loaded independent of DNA replication (160, 169).

The cohesin complex has been demonstrated to be localized to CCCTC-binding factor (CTCF) sites on human chromosomes, indicating a role for cohesin as a transcriptional insulator. Cohesin is directed to consensus binding sites by the insulator protein CTCF, however the insulator activity of the complex requires

the presence of cohesin, possibly without the establishment of cohesion (177). Cohesin has also been reported to associate with CTCF on lytic control regions of Kaposi's sarcoma herpes virus (KSHV) to repress their transcription, and loss of CTCF leads to episomal instability in cell culture systems (155).

Cohesin is removed from chromatin through two mechanisms (98, 174). The majority is released from the chromatid arms by a cleavage-independent mechanism during prophase. The remaining cohesin, localized to the centromeres, is removed by the degradation of securin by APC/C at metaphase, resulting in the release of separin which cleaves Scc1 at anaphase onset (see Figure 2.2).

Figure 2.2 Cohesin in the Cell Cycle.
A timeline of the removal of cohesin from sister chromatids during mitosis. Waizenegger 2000 (174).


PROPHASE PATHWAY
ANAPHASE PATHWAY
APC
?
SEPARIN
no SCC1 cleavage
SCC1 cleavage
condensation
separation
decondensation
prophase
metaphase
anaphase
telophase

ChlR1

hChlR1 is the human homologue of the yeast helicase, CHL1 (5). In budding yeast CHL1 was shown to be required for sister chromatid cohesion (150). Knockout of *ChL1* in yeast results in a mis-segregation phenotype characterized by loss of specific chromosomes and chromosome non-disjunction (47). Work with mammalian ChlR1 has shown that a homozygous knockout in mice is embryonic lethal, with a high percentage of the cells displaying aneuploidy, a decrease in chromosomal cohesion and an increase in mis-segregation (73). Work in this lab has shown that knockdown of hChlR1 leads to lack of cohesion in cell culture resulting in a prolonged mitosis, with cells stalled at pro-metaphase, and eventual mitotic failure (124). Parish, *et al* also showed that ChlR1 can be found in complex with the cohesion proteins Smc1, Smc3 and Scc1.

ChlR1 was of interest to this lab as it was identified as an E2 binding partner in yeast two-hybrid assays, in work originated by Regina Park. Additional work by J.L. Parish has shown that E2 and ChlR1 co-localize in interphase cells and through prophase in mitotic cells. A point mutation in the transactivation domain of E2 (W130R) was able to abrogate E2 binding to hChlR1 and resulted in loss of E2 from mitotic chromatin in transfected cell lines. Knockdown of ChlR1 using siRNA also resulted in dissociation of E2 from cellular chromatin. Interestingly this point mutation did not affect binding of E2 to Brd4, nor did knockdown of this Brd4 result in loss of E2 association with mitotic chromatin.

Taken together this showed that ChlR1 is required for mitotic segregation of E2 (123).

This led to the question, since ChlR1 binds to cohesin and is required for mitotic segregation of chromosomes, is there a role for the cohesin proteins in partitioning of the papillomavirus genome?

Materials and Methods

Cell Culture. Scc1 myc HeLa cells were a generous gift from J.M. Peters. pMEP CV-1 FLAG HPV11 E2 and FLAG HPV16 E2 were a generous gift from A. McBride. hScc1 was cloned into the pTRE2 inducible vector system. Scc1-myc HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad CA.) with 10% fetal calf serum (Atlas Biologicals, Fort Collins CO), Pen/strep 100U/ml (Invitrogen), G418 200 μg/ml, hygromycin 200 μg/ml. Expression of hScc1-myc was induced by the addition of doxycycline to a final concentration of 2 μg/ml for 48 hours at 37 ºC (64). ID13 and pMEP CV-1 cells were cultured at 37°C in Dulbecco's Modified Eagle Medium (Gibco) with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml) in 5% CO_2. 1.4 X 10^6 ID13 cells were plated in a 10 cm dish and 24 hours later were synchronized using 5 mM thymidine (Sigma) for 12 to 16 hours (G1/S) or 100 ng/ml nocodazole for 12 hours (M). pMEP cells were induced to express E2 by the addition of 1 μM $CdSO_4$ for 4.5 hours. hTERT-RPE1 cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum, and 100 units/ml penicillin and 100 μg/ml streptomycin.

Immunoblot and Immunoprecipitation. Cells ($2x10^6$/10 cm dish) were rinsed two times with sterile PBS and then with 1 ml cold PBS containing 1 mM

dithiothreitol (DTT), and 1 mM PMSF was added to each plate. Cells were scraped into a 1.5 ml tube and centrifuged at room temperature 1000g for 3 minutes. Supernatant was removed, and pellets were used immediately or frozen at -80 ºC for up to one week. Frozen cells were thawed on ice for 20 minutes, the lysed in 200 µl lysis buffer (50 mM Tris-HCl [pH 8.0] 100 mM NaCl, 20 mM NaF, 10 mM KH_2PO_4, 0.1 mM DTT, 1% Triton X-100, 10% glycerol, Roche complete protease inhibitors, 1mM PMSF), incubated on ice for 30 minutes then centrifuged at 13000 g for 10 minutes at 4ºC to pellet debris. Samples requiring DNAse digestion were treated with 0.02 U/µl RQ1 DNAse (Promega) for 30 minutes at room temperature. Protein concentration was then determined using BCA assay (Pierce). For Immunoblot (western), 20 µg protein was loaded into each well of an SDS poly-acrylamide gel (E2 10%; hScc1 8%; together 10%). For immunoprecipitation equal amounts of protein in lysis buffer were added to binding buffer (50 mM Tris-HCl [pH8.0], 100 mM KCl, 0.1 mM EDTA, 0.2% NP-40, 0.1% BSA, 2.5% glycerol, 2 mM DTT, 1mM PMSF, protease inhibitors) at a 1:1 ratio along with 10 µl protein A Sepharose beads (rabbit II-1), or 10 µl protein G Sepharose beads (9E10 Ab or B201/B202). Immunoprecipitate 2 hours, 4ºC with agitation. Immunoprecipitation was followed by three washes with washing buffer (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.5% NP-40, 2 mM DTT, 1 mM PMSF) at room temperature. Supernatant was removed and samples boiled in 20 µl 2X SDS-PAGE loading buffer (62.5 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 1% DTT, 0.02% bromophenol blue, 12.5 mM EDTA) and loaded onto a polyacrylamide gel. Following gel electrophoresis

samples were transferred onto PVDF membrane (Pall) at 23v for 1 hour. Membranes were blocked overnight in 5% milk, TBST at 4ºC. Primary antibody was incubated for 1 hour at room temperature with rocking (hScc1 is Rad21 Abcam AB992 1:2500, II-1 1:2500, B201/02 supernatant 1:50). Anti-rabbit HRP or anti-mouse secondary was diluted 1:10000 in 1% milk/TBS-T and incubated with the blot for 1 hour. This was followed by extensive washing with TBS-T and all blots were developed with Thermo (Pierce) SuperSignal West Dura fluorescence detection kit.

Chromatin immunoprecipitation (ChIP). ChIP was performed using a protocol modified from Upstate Cell signaling. HeLa and C33a cells were cross-linked by the addition of formaldehyde (Sigma) (final concentration of 1%) to cell culture media. Cells were incubated for 10 minutes at 37ºC, and washed with cold phosphate buffered saline (PBS), and scraped into a 1.5 ml collection tube. Nocodazole synchronized cells were collected using mitotic shake-off washed with cold PBS and centrifuged at 1000 g. Cells were resuspended in 250 μl lysis buffer (1% sodium dodecyl sulfate (SDS), 10mM EDTA and 50 mM Tris-HCl, pH 8.1) and incubated on ice for 10 minutes. Lysates were sonicated for 50 seconds in 10-second pulses at 30% output (Fisher Scientific dismembrator), then centrifuged at 14000 g for 10 minutes at 4°C. The supernatant was then removed and diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1 mM PMSF, Roche complete protease inhibitor). The ChIP lysate was pre-cleared by the addition of

30 μl protein A agarose bead slurry (protein G for mouse monoclonals) (Upstate Cell Signaling) and incubation at 4°C, with agitation, for a minimum of 1 hour. After centrifugation the supernatant was removed and added to 50 μl fresh beads containing 5 μl II-1 rabbit anti-E2 antibody, 5 μl rabbit pre-immune serum, 3 μl 9E10 Ab (Santa Cruz), or 20 μl monoclonal B202 supernatant. Following overnight incubation at 4°C beads were pelleted at 4000 rpm and washed with cold low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris HCl, pH 8.1), and twice with cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Complexes were then released from the beads using one-15 minute incubation with 100 μl elution buffer (1% SDS, 0.1M $NaHCO_3$). The eluates were reverse crosslinked by incubation with NaCl (final concentration of 0.2M) at 65°C for 4 hours. Samples then were digested with 20 μg proteinase K (Invitrogen) at 45°C for 1 hour with the addition of EDTA to 0.01M and Tris HCl, pH 6.5, to 0.04 M. DNA was recovered using QIAquick PCR purification kit (Qiagen). DNA was re-suspended in water and analyzed by PCR.

***In vitro* binding assays** (contributed by J.L. Parish)

GST E2TAD, E2R and GST were expressed and purified as described by Yao, et al. (188). Smc1, Smc3, Scc1 cDNAs were amplified from a HeLa cDNA library and cloned into pcDNA3. Proteins were *in vitro* translated using either rabbit

reticulocyte lysate or wheat germ extract TNT kit (Promega) in the presence of ^{35}S-methionine (Perkin Elmer). For removal of DNA, samples were treated with 0.02 U/μl DNase RQ1 (Promega) for 20 minutes at 37ºC. 2 μg of purified GST or GST-E2 proteins bound to 20 μl of 50% glutathione Sepharose slurry were incubated with 10 μl radiolabeled proteins in binding buffer (100 mM Tris-HCl [pH 7.9], 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 0.02% BSA) for 90 minutes at room temperature with agitation. Beads were washed three times (100 mM Tris-HCl [pH 7.9], 150 mM NaCl, 0.1% NP-40, 1 mM DTT), separated by SDS-PAGE and quantified using a PhosphorImager (Fuji) and ImageQuant software.

Antibodies. II-1 Ab used 3 μL for co-IP; B201/B202 20 μl supernatant; 9E10 1μl (Santa Cruz SC-40); Scc1 (Abcam Ab 992) 1 μl IP, 2 or 3μl ChIP. Anti-FLAG Ab was obtained from Sigma (monoclonal M2 F-3165).

PCR. Primers for the BPV LCR are 5'-aaagtttccattgcgtctgg-3' sense, 5'-gctttttctagttagctggctatttt-3' antisense, with an annealing temp of 54ºC. The average number of cycles required was 25.

pBABE-puro primers are 5'-gtcaccgagctgcaagaact-3' sense and

5'-caggaggccttccatctgt-3' anti-sense.

Results

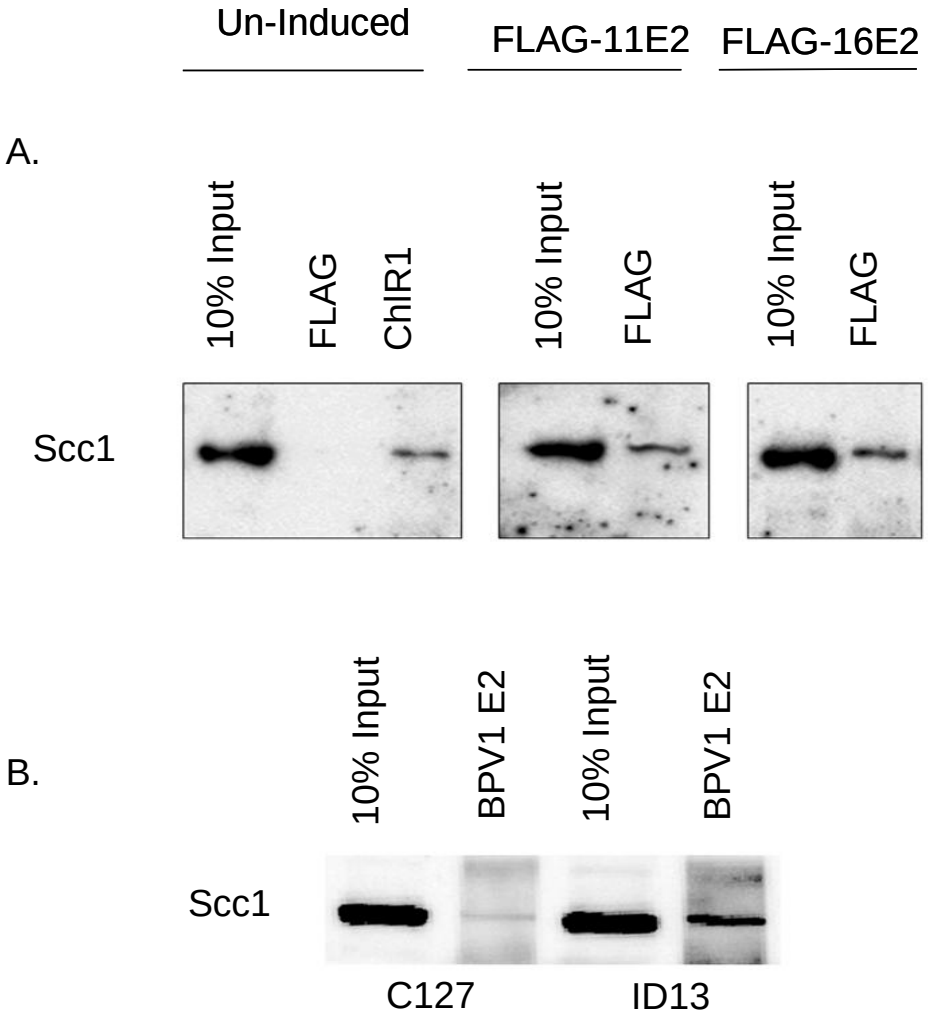
E2 and ChlR1 were found to be binding partners, and ChlR1 was found to bind to members of the cohesin complex, this led us to examine cohesin proteins to see if they, in fact, bound to E2. In work performed in this lab by Angela M. Bean, FLAG-HPV11 and FLAG-HPV16 E2 were induced in the pMEP cell line, co-immunoprecipitated using anti-Flag antibody, and immunoblotted for the cohesin protein Scc1 (Figure 2.3). A small percentage of Scc1 clearly co-immunoprecipitated with FLAG-E2 (2.3.A). To examine whether the interaction was detectable within a system expressing physiological levels of E2, the co-IP was again performed on the ID13 murine cell line which stably maintains the BPV1 genome. Scc1 clearly co-immunoprecipitated with endogenous E2 in ID13 cells and not in the parental C127 cell line that does not express the BPV genome. These results indicate that E2 binds to cohesin proteins in asynchronous cells when over-expressed and at lower endogenous levels.

To further explore the interaction between E2, hChlR1 and Scc1 the hScc1-myc HeLa cell line was obtained from the lab of J.M. Peters (64). These cells express an Scc1 construct containing 9 myc-epitope tags at the C-terminus directed by a tetracycline inducible promoter. Figure 2.4 illustrates detection of hScc1-myc in only the induced lanes. Antibody to endogenous hScc1 detects a band in both the induced and un-induced cell lines, with an additional band appearing above hScc1 corresponding to a higher molecular weight myc-epitope tagged hScc1.

Figure 2.3. E2 co-immunoprecipitates with Cohesin Proteins

A. pMEP-CV-1 cells that express FLAG-HPV11 or HPV16 E2 were induced or left un-induced and immunoprecipitated for FLAG with the M2 antibody (Sigma) and probed for hScc1. ChlR1 (un-induced panel) antibody precipitates hScc1.

B. hScc1 co-immunoprecipitates with endogenous BPV1 E2 in ID13 cells but not the parental C127 cell line using the B201 antibody. All panels include 5% input.


Un-Induced
FLAG-11E2
FLAG-16E2
A.
10% Input
FLAG
ChIR1
10% Input
FLAG
10% Input
FLAG
Scc1
B.
10% Input
BPV1 E2
10% Input
BPV1 E2
Scc1
C127
ID13

E2 was transfected into hScc-myc HeLa cells to examine the interaction between E2 and hScc1-myc in this cell line in the absence of BPV1 genomic DNA. E2 co-immunoprecipitates the labeled hScc1 in transfected cells using two different antibodies to wild type BPV1 E2 (Figure 2.5). It appears there are also Scc1 degradation products present that do not precipitate. An additional background band is also present in both induced and un-induced cells (marked with an asterisk).

The HeLa cell line was next used to examine the interaction between hScc1 and BPV1 E2. Wild type BPV1 E2 (500 ng), expressed from the CMV promoter in a pCDNA3 construct, was transfected into HeLa cells (10 cm dish) 24 hours post-induction, along with the E2 repressor protein (E2R)(250 ng) and the E2 mutant that does not bind to ChlR1, E2 W130R (500 ng). The cells were harvested 24 hours later and co-immunoprecipitation experiments were performed with both E2 rabbit II-1 and 9E10 mouse antibodies (See Figure 2.6).

Figure 2.4 Detection of hScc1-myc in HeLa cells.
Following 48 hour induction hScc2-myc HeLa cells were lysed and Run on an 8% acrylamide gel. The left side was probed with monoclonal 9E10 myc-antibody (1:2500), the right with polyclonal Ab to hScc1 (1:2500). Both were incubated with their corresponding secondary antibody at a 1:10,000 dilution then developed with the Super Signal West Dura kit (Thermo/Pierce).

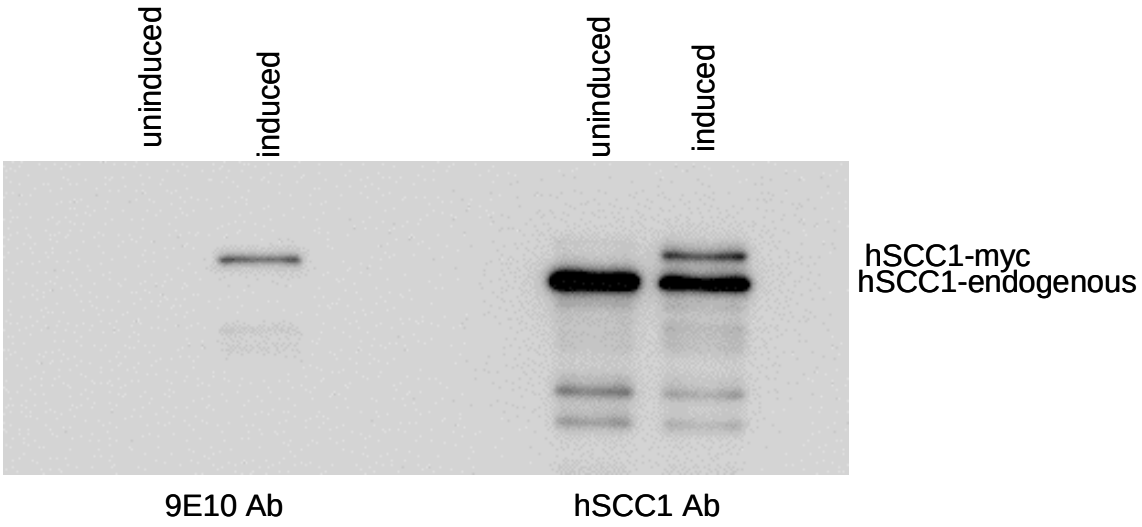
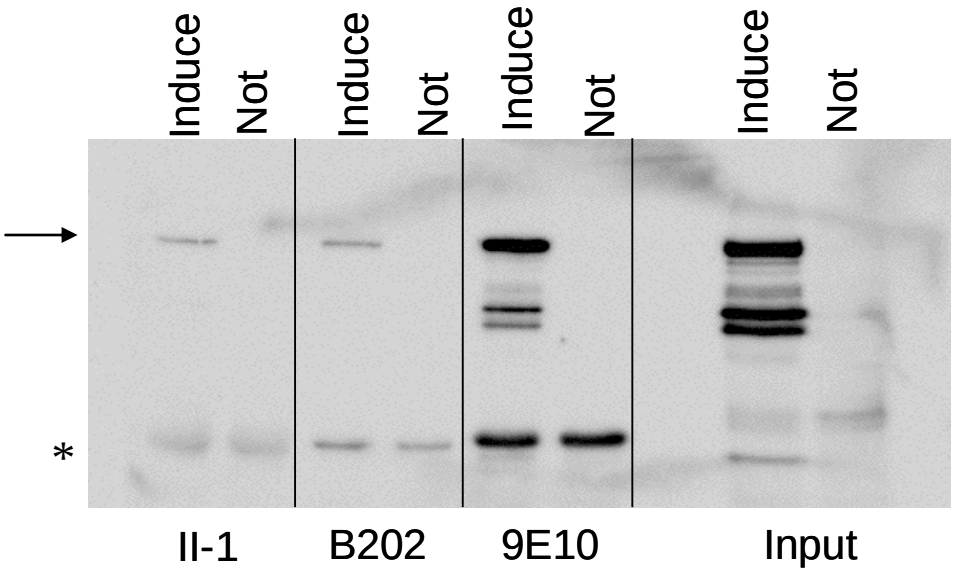
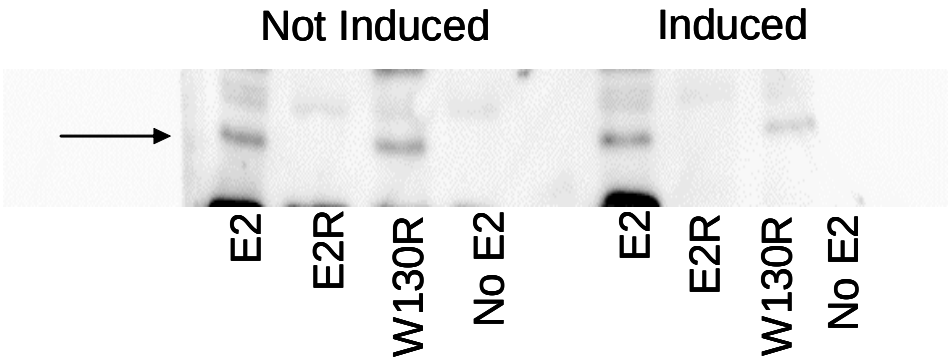

uninduced
induced
uninduced
induced
hSCC1-myc
hSCC1-endogenous
9E10 Ab
hSCC1 Ab

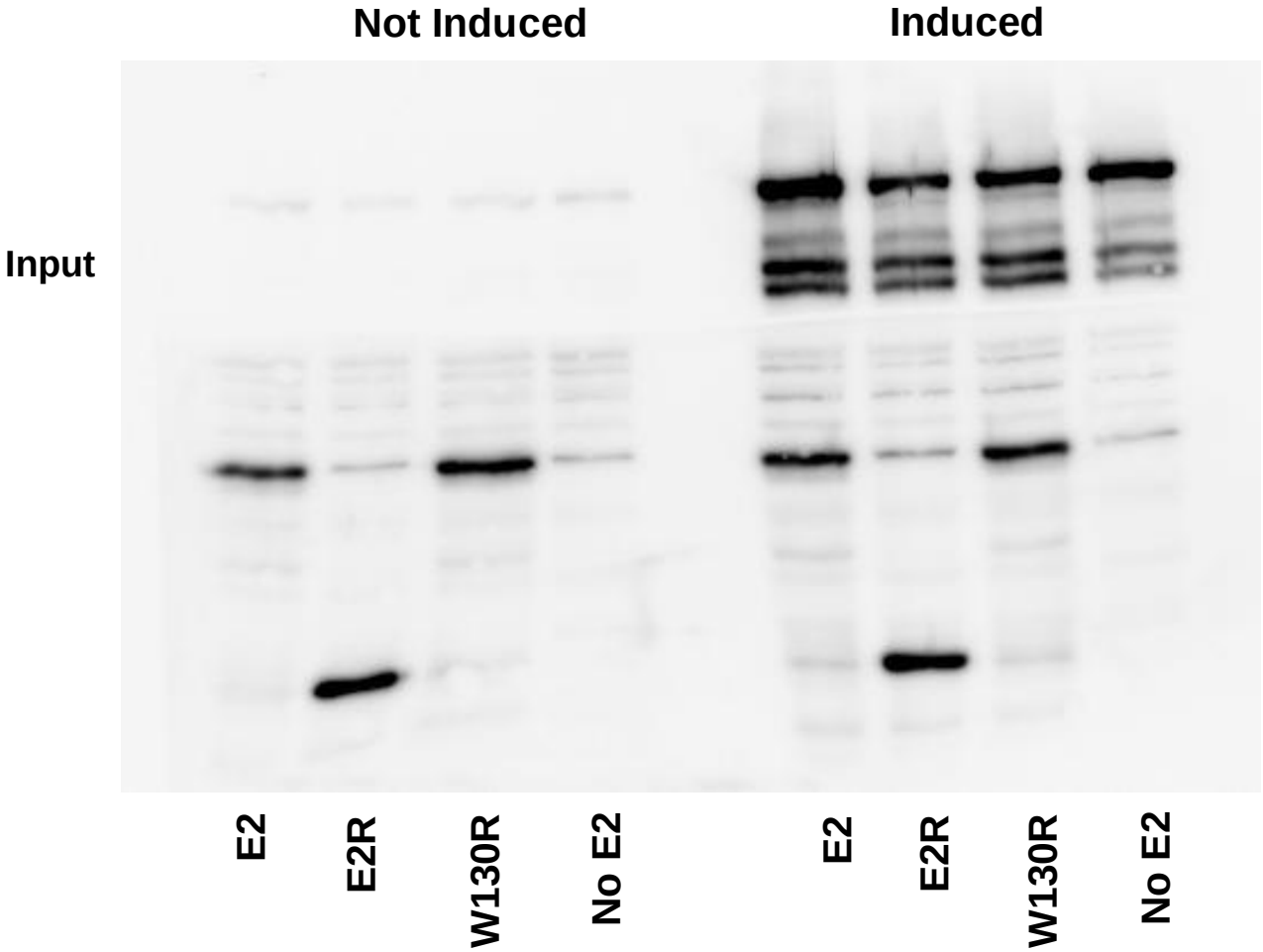
Figure 2.5. **Co-immunoprecipitation of E2 and HScc1-myc in HeLa cells**. hScc1-myc HeLa cells were induced to express myc-tagged hScc1 and immunoprecipitations were performed using antibodies to E2 (II-1 rabbit or B202 mouse) and myc (9E10). For each antibody the cells were induced with doxycycline 2 µg/ml 48 hours or were left untreated. The arrow indicates the position of full length hScc1. Immunobot was performed using 9E10 antibody and developed with Thermo Pierce Super Signal West Dura kit. 10% input is shown on right. The asterisk is an unidentified background band.


Induce
Not
Induce
Not
Induce
Not
Induce
Not
*
II-1
B202
9E10
Input

Co-immunoprecipitation of hScc1-myc with E2 and E2 with hScc1-myc both proved to be increasingly problematic. E2, E2R and E2W130R were immunoprecipitated with hScc1-myc and the reverse IP worked as well (not shown). The difficulties were primarily with the un-induced negative control cell lines. In all cases the immunoprecipitation detected the target proteins regardless of whether the cells had been induced (See Figure 2.6). This is possibly due to background expression which is visible, faintly, in the uninduced cells (See input panel below). It is also possible that there are additional proteins in complex with E2 that might react with the 9E10 antibody, the primary alternative being C-Myc itself. However, there was never a clear indication, in the form of a band in the 60-70 kDa range in a 9E10 blot, that C-Myc was present in the IP, aside from possibly the first IP with E2 (Figure 2.5 asterisk). Additional attempts were made to continue these experiments with the hScc1-myc HeLa cell line. The salt content of the wash buffer for immunoprecipitation was increased up to 500 mM, the beads were given an additional pre-block in 5% BSA or milk buffer, the amount of E2 was reduced to 500 ng/10 cm dish, and an additional DNAse digestion step was added prior to the immunoprecipitation in the event that all proteins were binding non-specifically to DNA. None of these strategies were successful in blocking the binding in the un-induced cell lines.

Figure 2.6. **Co-immunoprecipitaion of E2 and hScc1-myc in transfected HeLa cells.**
Induced and un-induced HScc1-myc HeLa cells are transfected with BPV1 E2, E2R or W130R immunoprecipitated with 9E10 antibody and blotted with anti-E2 II-1 (top panel). The arrow indicates the position of full length E2. The bottom panel depicts 5% input for both uninduced and induced cells. E2 was detected with the II-1 rabbit antibody.

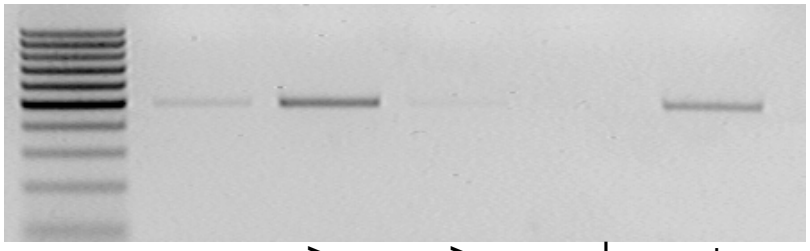

Not Induced
Induced
E2
E2R
W130R
No E2
E2
E2R
W130R
No E2


Not Induced
Induced
Input
E2
E2R
W130R
No E2
E2
E2R
W130R
No E2

In addition to co-immunoprecipitation assays, ChIP assays were also undertaken on the hScc-1 myc HeLa cell line in order to determine whether the tagged cohesin protein would interact with the BPV1 genome. Cells transfected with the BPV1 genome were induced at 24 hours post-transfection and then harvested for ChIP 48 hours later. The ChIP data correlated with expected expression of the inducible hScc-myc (Figure 2.7). However the BPV1 genome was detected in the un-induced HeLa cell line. BPV1 E2 expression was sufficient in these samples for ChIP of the viral genome using the II-1 antibody (Figure 2.7 lower panel). To determine whether the cohesin protein was binding to replicated DNA, a portion of the product of the ChIP assay was digested with *DpnI*. There is a restriction site for this enzyme located between the primers of set C. In Figure 2.8 the cohesin protein ChIPs the BPV1 genome without the DNA having replicated, as does BPV1 E2, evidenced by the lack of PCR product in DpnI digested samples. Scc1 is loaded onto the viral DNA, and it interacts with E2, but it is not clear whether the hScc1-DNA interaction is dependent on E2. To determine E2 dependence an empty vector and the BPV1 genome were transfected into the hScc-myc HeLa cells. The cells were induced as previously described and harvested for ChIP assay.

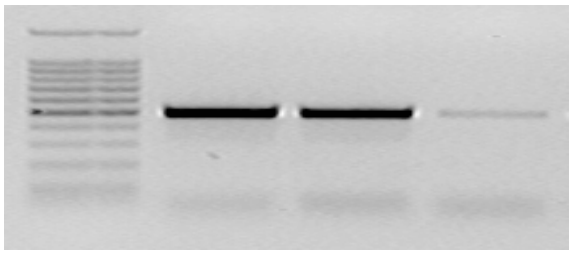
Figure 2.7 ChIP of the BPV1 genome transfected into hScc1-myc HeLa Cells.

Cells were transfected with 150 ng BPV1 genome, induced to express myc-tagged hScc1 24 hours post transfection and harvested 48 hours later. Top panel hScc1 ChIP for the viral LCR. Bottom panel is BPV1 E2 ChIP using II-1 A. Primer set C was used for PCR of all samples.



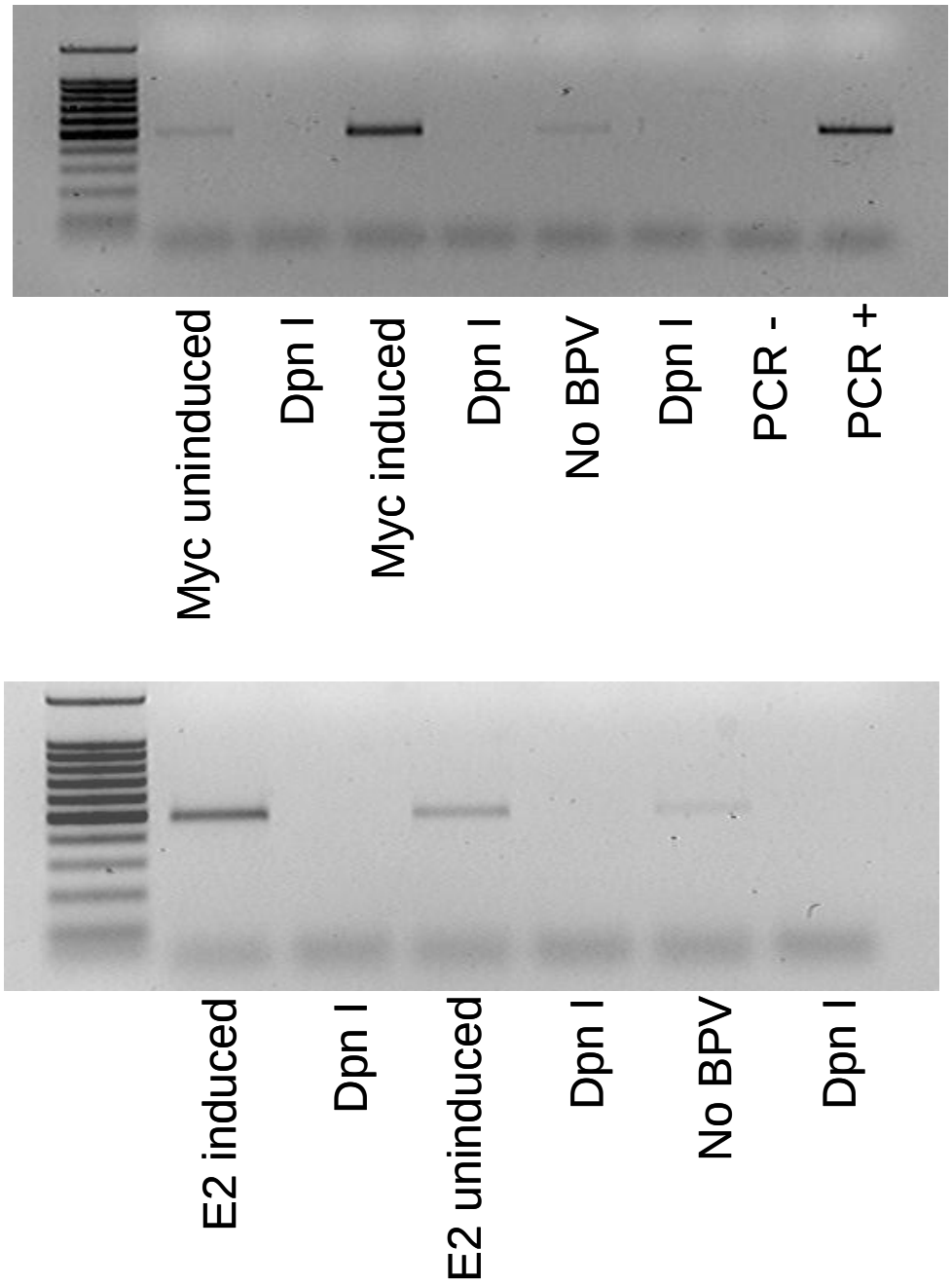
Uninduced cells + BPV
Induced cells + BPV
Induced cells No BPV
PCR-
PCR+

ChIP with 9E10



ChIP with II-1

Figure 2.8 ChIP and digestion of BPV1 genome in hScc1-myc HeLa Cells. hScc-myc HeLa cells were transfected with BPV1 genomic DNA and 24 hours later were induced to express hScc1 myc. At 72 hours post-transfection cells were harvested for ChIP assay. Samples were digested with DpnI or mock digested and PCR was run using primer set C. The top panel are results of ChIP with 9E10 for hScc1-myc, the bottom for E2 using the II-1 antibody.


Myc uninduced
Dpn I
Myc induced
Dpn I
No BPV
Dpn I
PCR -
PCR +
E2 induced
Dpn I
E2 uninduced
Dpn I
No BPV
Dpn I

hScc1 was induced as in previous experiments and harvested at 72 hours. The results are illustrated in figure 2.9. In addition to the 9E10 antibody, a commercial antibody to hScc1 was used (Abcam anti-Rad21 Ab992). PCR primers were designed for the pBABE-puro vector covering nucleotides (537-743). E2, hScc1-myc and hScc1 immunoprecipitated the BPV1 genomic DNA. Samples transfected with pBABE-puro only produced a PCR product in the presence of either the hScc1 antibody or the 9E10 monoclonal antibody. This data, combined with data from previous figures indicates that hScc1 will bind to the papillomavirus genomic DNA that has not replicated, and that binding to plasmid DNA is not dependent on the presence of the papillomavirus E2 protein.

Chromatin immunoprecipitation data in the inducible HeLa cells was collected from an asynchronous population. It is thought that cohesion is established in S-phase cells (168). To determine whether cohesion is established in the binding of cohesin to BPV genomic DNA, ChIP was performed in the mouse ID13 cell line synchronized by either the addition of 5 mM thymidine (G1/S) or 2 ng/ml nocodazole (M phase). Asynchronous cells were also included. Figure 2.10 is a graph of real-time PCR for primers in the LCR region showing E2 and hScc1 in both asynchronous and cycled cells. E2 can immunoprecipitate the genome readily in all phases of the cell cycle, with the strongest bands appearing in the asynchronous cells. ChIP of hScc1 appears to be cell cycle dependent with no binding in cells blocked at G1/S, some in asynchronous and the strongest binding occurring in cells blocked in early mitosis.

Figure 2.9 ChIP of E2, CTCF, hChlR1 and hScc1.
ChIP performed on hScc1-myc HeLa cells transfected with either the BPV1 genome or pBABE-puro. hScc1 antibody Abcam (Ab992). Input is diluted 1:10 for PCR.

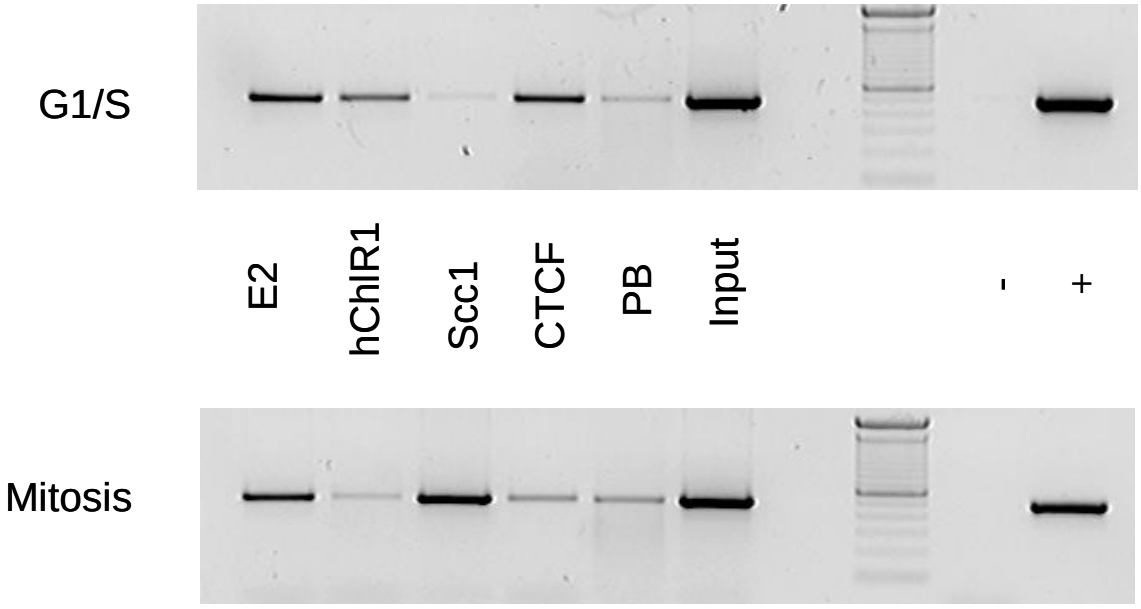
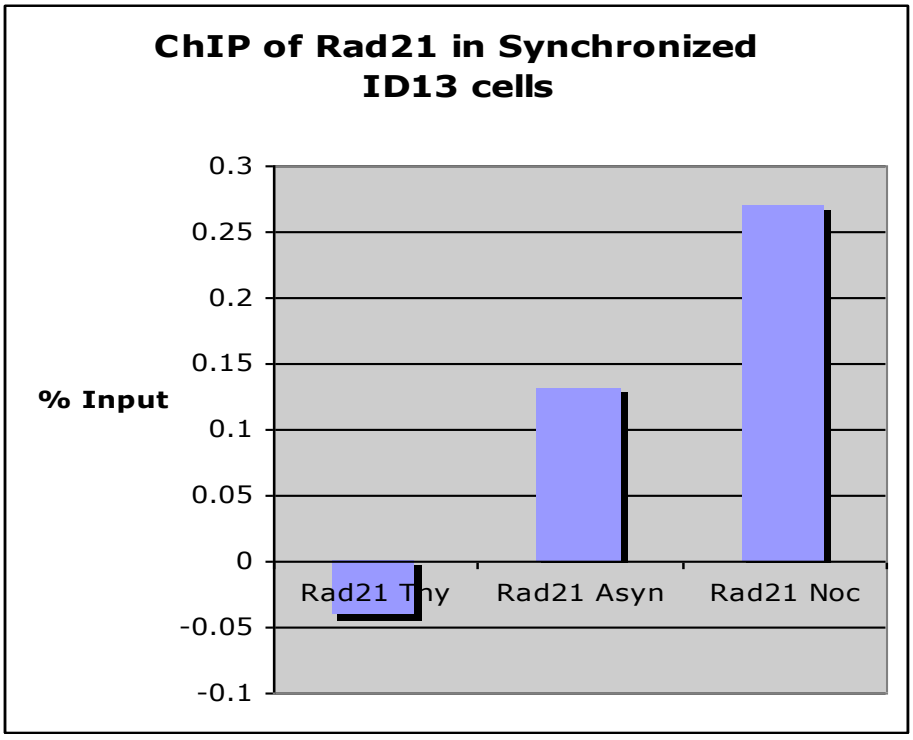

G1/S
E2
hChIR1
Scc1
CTCF
PB
Input
-
+
Mitosis

Figure 2.10 RT-PCR of ChIP samples.
The BPV1 genome was immunoprecipitated with either the antibody to E2 (II-1 rabbit) or hScc1 (Ab992) in ID13 cells blocked at G1/S (Thy), mitosis (Noc), or in asynchronous cells (Asyn). The binding is calculated as percent of input, after subtraction of PB (negative control) from each sample following 45 cycles of RT-PCR (MJ Research).


ChIP of Rad21 in Synchronized ID13 cells
% Input
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
Rad21 Thy
Rad21 Asyn
Rad21 Noc

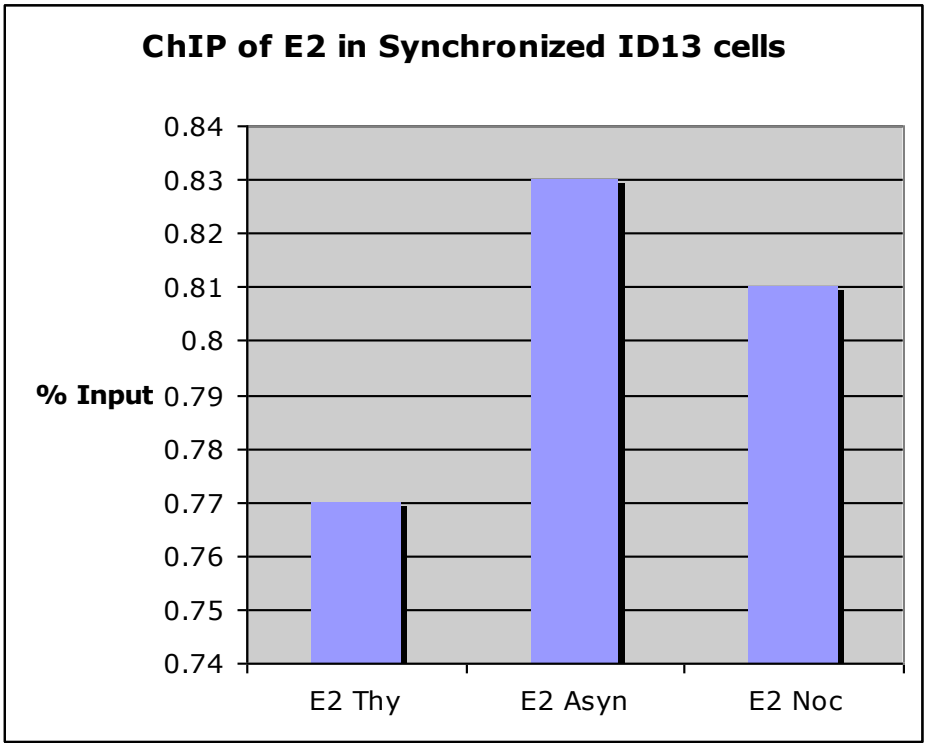
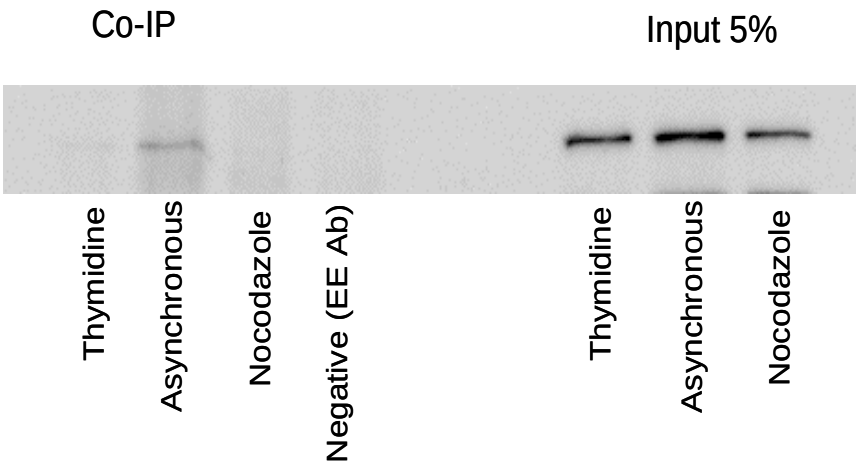
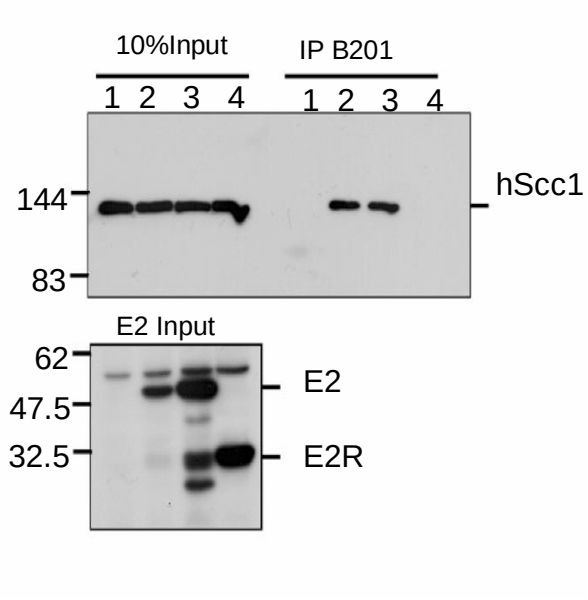

ChIP of E2 in Synchronized ID13 cells
% Input
0.84
0.83
0.82
0.81
0.8
0.79
0.78
0.77
0.76
0.75
0.74
E2 Thy
E2 Asyn
E2 Noc

Figure 2.11. Immunoprecipitation of E2 and hScc1 in ID13 Cells.
ID13 cells were synchronized with either 5 mM thymidine, 100 ng/ml nocodazole or were left asynchronous. Upon harvest lysates were immunoprecipitated with a mix of BPV1 monoclonal antibodies (B201/B202) and run on a 10% acrylamide gel, transferred to a PVDF membrane , and blotted for hScc1 using Ab992 (Abcam).

Bottom panel courtesy of J.L.Parish. C33a cells were untransfected (lane 1) transfected with BPV1 E2 (lane 2), E2 W130R (lane 3), or E2R (lane 4).



IP B201/02
Blot Scc1

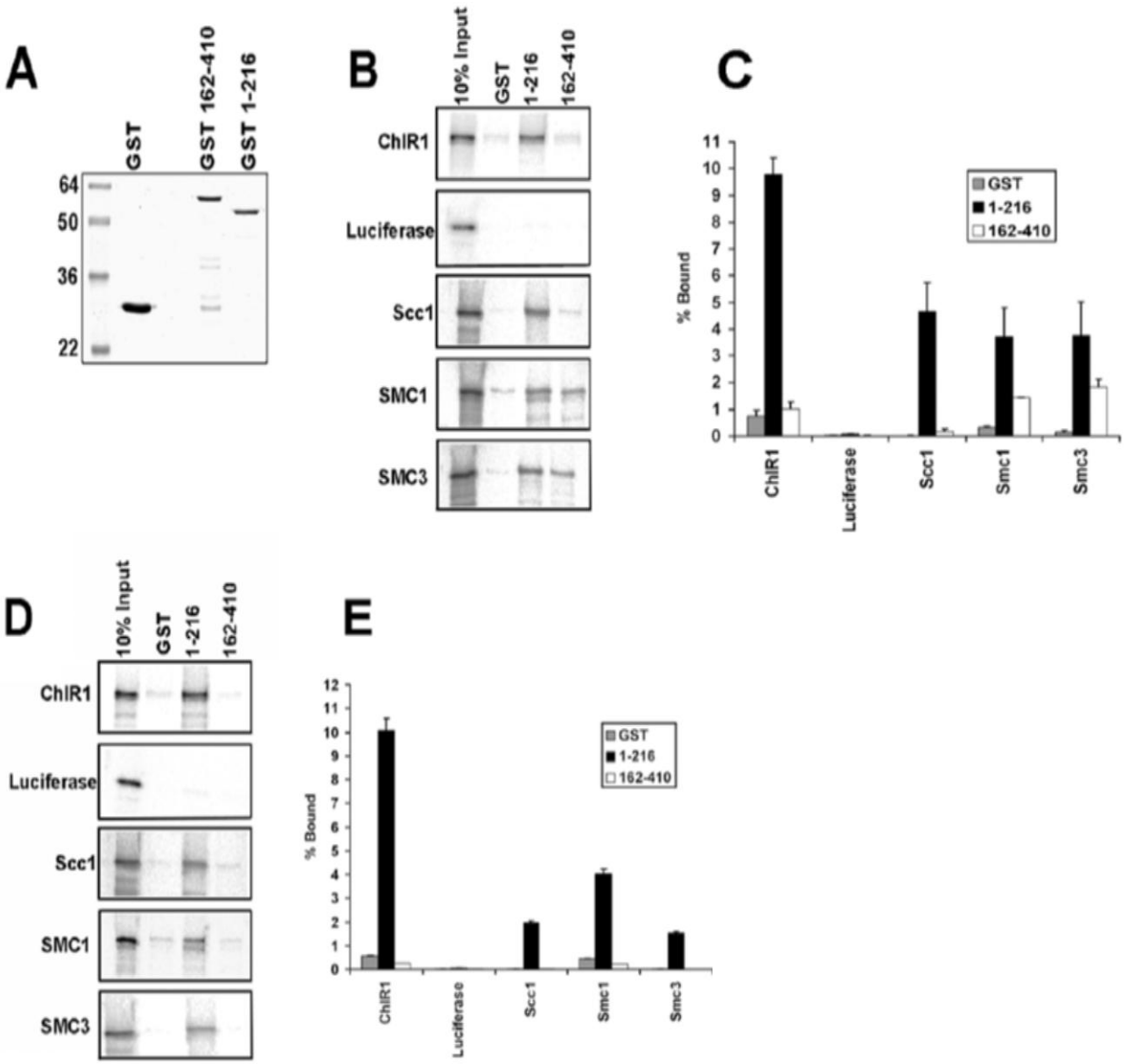


Based on the results of the ChIP data in synchronized cells, another experiment was undertaken to determine cell cycle dependence of the E2/hScc1 interaction. ID13 cells were synchronized and the E2 protein was immunoprecipitated with the monoclonal antibody B201. An immunoblot for hScc1 using the rabbit polyclonal antibody showed a co-IP of the cohesin protein only in asynchronous cells (Figure 2.11). In order to determine whether this interaction was dependent on ChlR1, an E2 mutant that does not bind ChlR1 was transfected into C33a and immuoprecipitated with the E2 monoclonal antibody B202. Both E2 and E2 W130R bind hScc1, suggesting that the interaction of E2 with cohesion proteins is independent of ChlR1 (Figure 2.11 bottom panel).

Dr. Joanna Parish did additional *in vitro* work to establish the regions of E2 that interact with the cohesin proteins. Nucleotides 1-216 and 162-420 of BPV1 E2 were generated as gst-fusion proteins and expressed in *E. coli*. Figure 2.12.A shows coomassie stain of the purified proteins. These mutants and gst expressed alone were mixed with [S^{35}] labeled proteins and precipitated with glutathione-Sepharose beads. The results are shown in figure 2.12.B. and quantified in 2.12.C. Both the transactivation domain of E2 (1-216) and E2R (162-410) bind to three cohesin subunits, Smc1, Smc3 and Scc1. Digestion with DNAse (figure 2.12.D., quantified in 2.12.E.) resulted in loss of the interaction between the cohesin proteins and E2R, indicating that the positive result here emanates from the interaction between these proteins and a DNA intermediary. An interaction does remain between the E2TAD and three proteins of the cohesin complex.

Figure 2.12 Mapped Binding of E2 with hScc1.

Courtesy of J.L. Parish. Figure 2.12.A. coomassie staining of gst-E2TAD (1-216), gst-E2R (162-410) and gst, purified from *E. Coli*. 2.12.B. Purified E2 on gst-Sepharose beads combined with *in vitro* [S^{35}]-methionine labeled cohesin proteins Smc1, Smc3 and Scc1 and separated using SDS-PAGE. Quantification (2.12.C) with ImageQuant software (Fuji) based on 10% input. 2.12.D. *In vitro* translations were digested with DNAse and repeated as described above, with quantitation in 2.12.E. hChlR1 used as a positive control for E2 binding, TnT luciferase as a negative.

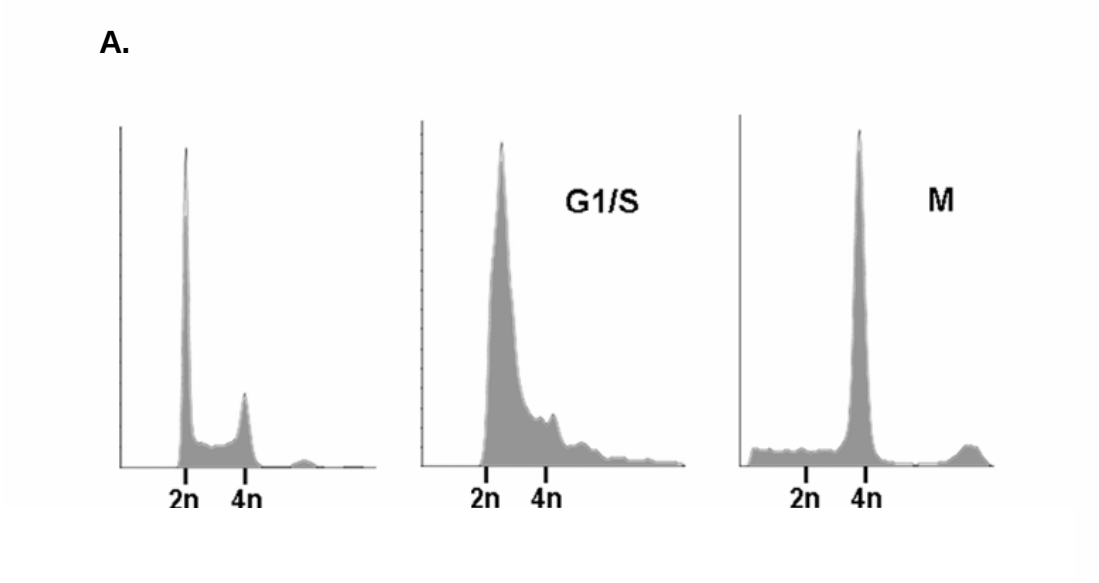

A
GST
GST 162-410
GST 1-216
64
50
36
22
B
10% Input
GST
1-216
162-410
ChlR1
Luciferase
Scc1
SMC1
SMC3
C
% Bound
GST
1-216
162-410
ChlR1
Luciferase
Scc1
Smc1
Smc3
D
10% Input
GST
1-216
162-410
ChlR1
Luciferase
Scc1
SMC1
SMC3
E
% Bound
GST
1-216
162-410
ChlR1
Luciferase
Scc1
Smc1
Smc3

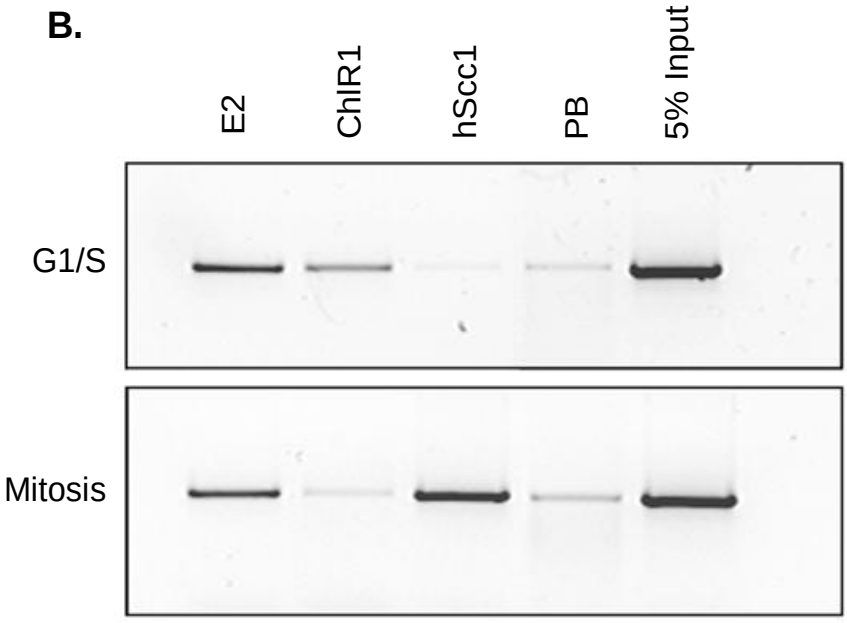
In an attempt to link hScc1 binding to the viral genome, and loading of cohesin by the ChlR1 protein, we analyzed the association of ChlR1 with the BPV1 episome in cycled ID13 cells. Cells were synchronized as previously described, and ChIP assays were performed with the II-1 rabbit anti-E2 antibody, the hScc1 rabbit polyclonal, and a goat anti-ChlR1 antibody developed in this lab (124). ChlR1 and E2 both bind to the viral genome during S phase (2.13.B. top panel). E2 and hScc1 are localized to the LCR at the onset of mitosis, but not in G1/S phase. The hScc1 data correlates with previous data obtained with hScc1 and the BPV1 genome in cycling cells (Figure 2.10), and the ChlR1 data is consistent with published data regarding dissociation of the protein from E2 and cellular genomic DNA in mitosis (123). In addition, hScc1 has been shown to co-localize with CTCF at regions of genomic and viral DNA. Preliminary data shown here suggest that CTCF may also serve an insulator role in regulation of papillomavirus proteins, as the BPV genome immunoprecipitates with antibody to CTCF.

Figure 2.13. ChIP of Cycled ID13 Cells.

A. ID13 cells are asynchronous (left panel), stalled at G1/S with 5 mM thymidine (center) or in mitosis with 100 ng/ml nocodazole (right).

B. ChIP of ID13 cells at G/S (top panel) or Mitosis (lower panel). Samples were immunoprecipitated with the II-1 E2 rabbit antibody, hScc1 (Ab 992), a goat anti-ChlR1, rabbit anti-CTCF (Bethyl Labs A300-543A) or pre-immune serum (PB). PCR primer set for the viral LCR 25 cycles.


A.
G1/S
M
2n
4n
2n
4n
2n
4n


B.
E2
ChlR1
hScc1
PB
5% Input
G1/S
Mitosis

Discussion

Previous work in this lab by Joanna Parish, *et al* has shown a requirement for ChlR1 in eukaryotic cells. The helicase is required for cohesion of sister chromatids and interacts with the cohesin proteins Scc1, Smc1, and Smc3 (124). Dr. Parish also demonstrated that ChlR1 is required for proper segregation of the papillomavirus genome in replicating cells. This was accomplished by the creation of a mutation in BPV1 E2 that abrogates the interaction between E2 and ChlR1, resulting in a loss of viral genomes in a stable genome maintenance assay (123). This posed the interesting question of whether the cohesin proteins are also required for long term maintenance of the papillomavirus genome in dividing cells. The facts shown here support, but do not prove, that the mechanism required for stable propagation of the BPV1 viral genome is tethering of the viral genome through cohesion, mediated by E2 and ChlR1.

Initial data prepared in this lab by Angela Bean was very positive, with hScc1 detectably interacting with E2 in an overexpression system. Follow-up experiments with endogenous E2 suggested that Scc1 does interact with BPV1 E2 in a system that stably replicates viral genomic DNA (Figure 2.3).

hScc1 is a protein that plays a vital role in sister chromatid cohesion, and thus perturbation of its expression in cell culture is not well tolerated. In an attempt to express tagged hScc1, several different constructs were made that contained an N-terminal tag (HA or FLAG) under the expression of a CMV promoter. None of these constructs were properly expressed in transfected cell

lines, although protein was seen in *in vitro* translations of the constructs (data not shown). A generous gift of the hScc-myc HeLa cells from J.M. Peters enabled us to examine some of target interactions in this system. Data acquired here, although not quantified, suggests that the amount of hScc1 expressed cannot exceed a threshold level, as stable expression from an exogenous source did not exceed endogenous expression (Figure 2.4 and data not shown).

The HeLa cell line was not ideal for use with co-immunoprecipitation and ChIP assays. The cross-reactivity with c-Myc or leaky expression gave constant high background in these experiments. Extensive immunoprecipitation assays revealed very slight evidence of leaky expression in immunoblots. However co-IPs were positive in both the induced and control cells, making any data retrieved subject to all possible interpretations. Although leaky expression was a possibility, the levels of tagged hScc1 protein seemed very low, which should not result in binding of equal strength to that in the induced cell line (Figure 2.6 and additional data not shown). As previously mentioned, techniques that should have decreased non-specific binding to either beads or antibody did not reduce background in this assay. This would suggest that the background binding is specific, and the result of binding of the antibody to the expressed myc-tagged protein or to the c-Myc protein. ChIP assay in BPV1 transfected cells (not shown) reveals c-Myc bound to the viral episome, possibly with E2 and or BPV1 E6. It has been shown that c-Myc increases replication of the SV-40 origin of replication in monkey cells, and can replace the function of Large-T antigen (67, 82). More recent work reveals that c-Myc interacts with the pre-RC and localizes

to origins of replication (35). Work is presently underway to determine the relationship between c-Myc and the papillomavirus genome.

ChIP assays with the 9E10 antibody in figures 2.7, 2.8 and 2.9 may be interpreted together. Data in 2.9 suggests that a possible feed back loop governs expression of endogenous hScc1. In cells that are not induced to express hScc1-myc, both the 9E10 antibody and the endogenous anti-hScc1 antibody detect the viral genome in a ChIP assay. Upon induction, there is little or no detection of endogenous protein on the viral genome. In order for this data to be meaningful, two conditions must be fulfilled. The first condition is that the hScc1 promoter must be regulated by a feedback loop. This is highly possible given that the cohesin complex is now being seen as a negative regulatory protein that can inhibit gene expression (155, 177). Excessive hScc-myc produced as a result of induction may diminish the amount of hScc1 transcribed from the endogenous promoter. The second condition is that the hScc1-myc tag interferes with access of the antibody to its epitope. hScc1 binding to the BPV1 genome is seen with anti-hScc1 in both figures 2.10 and 2.13, so this antibody is effective at ChIP of this protein on the viral genome. In Figure 2.9 this antibody works only in cells in which the tagged hScc1 is not induced. The hScc1 antibody was raised to the extreme C-terminus of the protein and it is possible the 9 myc tags placed here deny access of antibody to this region. If these two conditions are accepted, figure 2.9 can be interpreted to show that E2 and hScc1 bind to the BPV1 genome, while hScc1 additionally precipitates the empty

pBABE-puro vector. This would reveal that E2 is not required for hScc1 to interact with episomal DNA.

Figures 2.7 and 2.8 shed additional light on the requirements for hScc1 binding to episomal DNA. BPV1 can be immunoprecipitated with hScc1-myc in the HeLa cells (see Figure 2.7). This ChIP was digested with *Dpn1* and the results subjected to PCR for the viral LCR, which contains a *Dpn1* restriction site. Figure 2.8 illustrates that both E2 and hScc1 bind to transfected viral DNA that has not yet been replicated. This would not be unexpected, as although cohesion is established during S phase, cohesin can bind interphase DNA without necessarily establishing cohesion (23, 98, 168).

If cohesion is established on the BPV1 genome, it would be assumed that cohesin would be detectable on the viral genome during S phase. We examined cohesin on the viral genome of ID13 cells that stably maintain BPV and are stalled on the border of G1/S. hScc1 did not immunoprecipitate the genomes at this stage (figure 2.10 top panel). It did, however IP in both asynchronous and mitotic cells. It is also unexpected that ChlR1 and hScc1 ChIP in different phases of the cell cycle. The role of ChlR1 in the establishment of cohesion is still unclear. However, since cohesion can also be established in interphase to assist with DNA repair, it is possible that this mechanism is the one used to deposit cohesin on the BPV genome. We are currently working to ascertain the details of the mechanism involved, and whether the interphase interactions play a more significant role in genome tethering than originally thought.

The mutant W130R also does not perform as we would expect. If E2 binds to ChlR1 and this association is required for loading of cohesin onto viral DNA, then we would expect that a mutant that does not interact with ChlR1 would not interact with hScc1. Nonetheless, E2 W130R was detected with Scc1 in transfected C33a cells (figure 2.11). This suggests that the interaction of E2 with hScc1 does not require ChlR1.

The above mentioned data, along with the ChIP of CTCF with the viral genome (Figure 2.13) might also suggest an additional role for cohesin in the papillomavirus life cycle. In addition to a possible role in tethering of the viral genome during mitosis, cohesin could be responsible for repressing viral gene expression. Work in the lab is ongoing to determine whether predicted insulator sites in the pre-E5 and L1 coding sequence of BPV1 are valid targets of CTCF and cohesin, and whether they are functional insulator regions.

Conclusions

The bovine papillomavirus E2 protein was shown here to remain localized to the E2 consensus binding sequences in the viral LCR throughout the cell cycle. Clustered E2 sites upstream of the viral origin maintain E2 during viral replication. It does not appear that E2 binds in stages, i.e. the protein is not found solely on the origin during replication, following which it leaves the genome and then is found only on the MME during mitosis. The BPV1 MME maintains E2 even when these binding sites are not serving to assist in segregation of the viral genome, and while those sites appear to be constantly filled, sites outside the LCR seem to rarely draw E2 in monolayer culture. E1 does not associate with E2 at consensus sequences away from the origin of replication, as it immunoprecipitates only with DNA in the immediate vicinity of the initiation site.

ChIP with restriction enzyme digest, RED-ChIP, is a particularly useful combination of techniques that assist in analyzing adjacent genomic regions that are conveniently separated by a restriction sequence. It enabled the examination of distinct segments of a compact portion of the viral LCR which would not have been discernable using a standard ChIP assay.

E2 mediated genomic segregation of the BPV1 episome has been shown to require ChlR1. Clarification of the mechanism through which this occurs implicates the cohesin protein complex. Cohesin can be detected binding to E2 through the TAD in asynchronous cell culture independently of ChlR1 and to the viral genome at the onset of mitosis. ChlR1 and the cohesin complex

perceivably ChIP on the viral genome at different stages of the cell cycle. Cohesin binds to the genome without a requirement for E2, and can associate with viral and plasmid DNA that has not replicated.

The insulator protein CTCF binds the viral genome and ChIPs in cells blocked at G1/S, but has been removed from the DNA by early mitosis.

Future Directions

Continued work is needed on the occupation of E2 recognition sequences in the viral genome. Several of these are located outside the viral LCR, have extremely low affinity for E2 and have not been shown to be occupied by the protein. It is important to know if E2 associates with these regions at any point in the viral life cycle, which can be mimicked in cell culture by differentiation of cell lines stably expressing the BPV1 genome. A switch in the usage of these regions may be the result of, or initiate, high output viral DNA replication found in differentiated keratinocytes. Use of re-ChIP might also be re-visited to determine whether E2 is actually present at the origin when E1 is present.

This work inadvertently led to intriguing questions regarding the role of c-Myc in BPV1 replication. Does Myc interact with E2, and if so does it have a role at the origin of replication, in the presence or absence of E1 and E2? E6 and c-Myc interact, as do E6 and E2. Does E6 have a role in initiation of replication *in vivo* and what would that be? Work on this project is now on-going in the lab. ChIP and co-immunoprecipitation experiments are being combined to answer these questions. This lab has a large panel of mutants of both E2 and E6. These could help determine the functional relationships of these proteins with c-Myc in the replication process.

Does papillomavirus subvert the replication licensing requirements of the cell, and if so how? What cellular proteins are required to launch genome duplication in cell culture and which are not? If not is there a viral gene that fills

the requirement for the cellular component or an additional method to avoid activation of cell cycle checkpoints? We now have an increased ability to consider the option of mass spectrometry for identification of proteins. This might be a viable alternative here to identifying each origin protein using antibodies and immunoblotting.

Both the cohesin complex and hScc1 bind to E2 and to the viral genome and may be involved in several facets of the viral life cycle. The roles of these proteins in transcription, tethering and DNA repair need to be further evaluated. Identification of the mechanism behind the interaction between the viral genome and cohesin might lead to a more in depth understanding of cohesin and cohesion in the mammalian system. This includes the establishment of cohesion versus the binding of cohesin and the role of ChlR1 in loading of the cohesin complex. Both replication dependent and independent methods might factor into the viral life cycle. E2 is required for proper distribution of the viral genome to sister chromatids. How does it regulate cohesin deposition and interaction with hChlR1?

The CTCF protein interacts with hScc1 and the BPV1 genome. Is it localized to the putative insulator sequences determined by the computer algorithm (insulatordb.utmem.edu)? Do the proteins co-localize to these sequences? Does removal/disruption of these sequences affect expression of early or late viral genes, and are these sites regulated in monolayer and differentiated cell culture? Many of these questions are currently under investigation in the Androphy lab.

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